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by

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Signed.....

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ABSTRACT

There is worldwide concern over the possible estrogenic effects of organic chemicals on a variety of wildlife and indeed on humankind. In the U.K., estrogenic compounds in sewage treatment works (STW) effluents have been implicated in causing the increases in egg yolk protein production observed in caged male trout and other fish species. At the initiation of the present study, few of the estrogenic compounds in STW effluents had been recognised, although circumstantial evidence suggested that steroidal hormones were primary candidates.

Cholesterol is abundant in STW effluents and is the precursor of all steroidal hormones biosynthesised in mammalian systems. Thus, the aim of the present study was to examine the hypothesis that cholesterol might undergo A-ring aromatisation, during sewage treatment, producing estrone and 17β-estradiol *via*, intermediates such as 19-norcholest-1,3,5(10)-trien-3-ol (NCT).

To study this hypothesis NCT was first synthesised *via* a known route and several of its chromatographic and mass spectral properties established for the first time. NCT itself was found to possess some estrogenic potential determined using an established assay but this was rather weak compared to 17β-estradiol – about 200,000 times less active at the minimum concentration needed to invoke a response. NCT also proved to be a much more hydrophobic compound than, for example, 17β-estradiol with a computed log octanol-water partition coefficient (K_{ow}) of over 9 compared with a log K_{ow} of about 4 for 17β-estradiol. The established analytical properties of NCT were then used to investigate possible NCT formation in sewage.

Radiolabelled ^{14}C-cholesterol was incubated aerobically and anaerobically in Semi-Continuous Activated Sludge (SCAS), Die Away (DA) or simple stand alone STW simulation vessels. The products of incubation in both aqueous and solid fractions were examined by radio-high performance liquid chromatography (r-HPLC), radio-thin layer chromatography (r-TLC) and radio-gas chromatography (r-GC). Aerobic studies showed that side chain cleavage and A-ring rupture of cholesterol occurred rapidly (~25 % of added activity within 24 hrs) as measured by $^{14}CO_2$ evolution. Gaseous evolution was not monitored from the anaerobic experiments. Most remaining activity was associated with the solids fractions in all experiments. In the aqueous experiments both SCAS and DA systems, r-HPLC revealed rapid production of polar products which were not identified further. r-HPLC also revealed non-polar components of which cholest-3,5-diene, an unknown cholestadiene, a cholestadienol (other than 5,7-dienol), cholest-4-en-3-one and possibly NCT were identified by r-GC in the products of DA experiments. Whilst r-HPLC and r-TLC also revealed several products of anaerobic digestion of cholesterol, no compounds were detected by r-GC.

STW effluents from two wastewater plants in the North London area were monitored over 7 months for A-ring steroids and other suspected estrogenic chemicals. Both effluents had previously proved estrogenic to caged fish. Liquid and SPM samples were taken, extracted and analysed by GC-MS. The two main estrogens, 17β-estradiol and estrone were identified from all liquid samples but not in SPM extracts. Generally the concentration of estrone (maximum *ca* 3 ng L^{-1}) was significantly higher than that for 17β-estradiol (maximum *ca* 1 ng l^{-1}). The third natural estrogen, 16α,17β-estriol, was identified in all Harpenden effluent samples analysed up to a concentration *ca* 0.5 ng L^{-1}. However, estriol was not found in Deephams effluent extracts. The phytoestrogen, daidzein, found in soya based products was intermittently found in aqueous effluents samples from both sites >1 ng L^{-1}. SPM made up <0.001 % of the effluent. Extracts showed that there was a high percentage of steroidal based material with coprostanol>cholesterol≈β-sitosterol>>stigmasterol. NCT was twice identified from SPM taken from Deephams with a concentration of 39 and 136 ng l^{-1}, but <LOD in Harpenden SPM extracts.

In conclusion this study showed that formation of NCT from cholesterol in STW treatment under conditions monitored herein is not a major process. However, estrone, 17β-estradiol and 16α,17β-estriol are relatively abundant and widespread components of the STW effluents tested. The origins of the components undoubtedly include direct inputs from female urine and faeces but other metabolic sources should not be entirely ruled out.

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Chapter 3

Synthesis of a putative intermediate in the bacterially-mediated conversion of cholesterol to estrone: 19-norcholest-1,3,5(10)-trien-3-ol (NCT)

Chapter 4

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Chapter 5

Monitoring of selected estrogenic compounds and other sterols in STW effluents

Chapter 6

Authors Declaration

At no time during registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

Relevant scientific seminars and conferences were attended at which work was often presented.

Presentations and Conferences attended;

Niven, S.J., S. Rowland, J. McEvoy, M. Depledge, J. Snape, M. Evans and M. Hetheridge Determination of the Possible Estrogenic Degradation Products of Cholesterol in Sewage Treatment Works. (Poster presentation). SETAC-Europe meeting, Bordeaux, France. April 1998.

Niven, S.J., S. Rowland, J. McEvoy, M. Depledge, J. Snape, M. Evans and M. Hetheridge Determination of the Possible Estrogenic Degradation Products of Cholesterol in Sewage Treatment Works. (Poster presentation). 10th Annual Meeting of the British Organic Geochemical Society, University of Plymouth, July 1998.

Niven, S.J., S. Rowland, J. McEvoy, M. Depledge, J. Snape, M. Evans and M. Hetheridge Examining the Estrogenic Potential of Sewage Treatment Works Effluents. (Oral presentation). University of Plymouth, January 1999.

Niven, S.J., Snape, J., Hetheridge, M., Evans, M., McEvoy, J., Sutton, P.G. and Rowland, S.J. (1999) Investigations of the origins of estrogenic A-ring aromatic steroids in sewage treatment works effluents. In *Endocrine Disrupters: The Analytical Challenge*, held at Warrington, 11-12 October 1999. Special Publication Royal Society of Chemistry. (In press).

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Date 28th June 2000

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List of Common Abbreviations

ARC	Authentic reference compound
BSTFA	*N,O*-Bis(trimethylsilyl)trifluoroacetamide
CPS	Counts per second
DA	Die Away
DCM	Dichloromethane
EPA	Environment Protection Agency
GC-FID	Gas Chromatograph-Flame Ionisation Detector
GC-MS	Gas Chromatograph-Mass Spectrometer
GF	Gas Fibre
H	hours
HP	Hewlett-Packard
HPLC	High Performance Liquid Chromatography
LSC	Liquid Scintillation Counting
ODS	Octadecasilane
PTFE	Teflon
RD	Radioactivity detector
RF	Response factor
rGC	Radio Gas Chromatography
rHPLC	Radio High Performance Liquid Chromatography
RI	Retention index
RPM	Revs per minute
RT	Retention time
rTLC	Radio Thin Layer Chromatography
SCAS	Semi-continuous activated sludge
SPE	Solid Phase Extraction
STW	Sewage treatment works
THF	Tetrahydrofuran
TIC	Total ion current
TLC	Thin Layer Chromatography
TMCS	Trimethylchlorosilane
TMS	Trimethylsiloxane
Vg	Vitellogenin

Chapter 1

General Introduction

There is currently world-wide concern over the so-called "environmental estrogenic" effects of many organic chemicals, both natural and anthropogenic. Numerous articles have reported these phenomena in the press (*e.g.* Bower, 1997), in popular scientific journals (*e.g.* McLachlan and Arnold, 1996; Stone, 1994) and in two best-selling books (Colburn *et al.*, 1996; Cadbury, 1997). Environmental, medical and health aspects of estrogenicity have been reviewed. Essentially, the major concerns are the world-wide lowering of human male sperm count, the rise in certain cancers such as breast and testicular cancer and the numerous observations of wild animals showing imposex, feminisation, masculinisation or reproductive depression. Given the broad range of effects loosely classified as "estrogenic" it is worth examining what is meant by the term "estrogenic" and how such effects are assayed.

1.1 What is meant by estrogenic?

Estrogenic : chemicals that induce estrus, applied to hormones; pertaining to estrogen (Henderson, 1995).

Estrus : the period of sexual heat and fertility in a female mammal when she is receptive to the male (Henderson, 1995).

Natural steroidal estrogens are by definition termed "estrogenic" and hence non-steroidal estrogens that induce the same biochemical changes as natural estrogens are also termed "estrogenic". Non-steroidal estrogens work by mimicking the role of natural steroidal estrogens by binding to the estrogen receptor and triggering a

response. Such estrogen mimicking compounds are called xenoestrogens. Not all chemicals that bind to the estrogen receptor trigger an effect. If a chemical binds tightly to the estrogen receptor but does not induce an effect the receptor is unavailable. When a number of receptors are 'blocked' in this manner there is a lowered estrogenic effect, which indirectly causes an anti-estrogenic effect.

1.1.1 Determination of estrogenicity

Chemicals suspected of being estrogenic can be tested *in vivo* and *in vitro via* a number of different methods (Table 1.1). *In vitro* - biological reaction or processes taking place in extracts from tissues or cells. *In vivo* – biological reactions or processes taking place in a living cell or organism. *In vitro* studies enable a rapid, relatively cheap assay of test compounds often prior to confirmational *in vivo* studies, where these are deemed necessary.

Assay	**Application**
Yeast Assay (YES)	*in vitro*
Vitellogenin assay	*in vivo / in vitro*
Breast cancer cell assay (E-screen assay)	*in vitro*
Direct Observation	*in vivo*

Table 1.1 Assays for determination of estrogenicity and their application mechanism.

1.1.2 The yeast assay

The yeast assay uses a human estrogen receptor gene incorporated into the yeast chromosome (Routledge and Sumpter, 1996). The activity of the receptor is measured by the production of the enzyme β-galactosidase secreted from the yeast cell. β-galactosidase interacts with chlorophenol red-β-D-galactosidase indicator (yellow) to produce a red colour of which the absorbance can be measured (Routledge and Sumpter, 1996). The genetically modified yeast is cultured and stored for a maximum of 4 months (Routledge and Sumpter, 1996). To demonstrate the assay, authentic reference compounds were dissolved in ethanol, spotted onto wells of a 96 well micro plate and blown down to dryness. To the reference compounds 200 μL of yeast medium containing chlorophenol red-β-D-galactosidise was added. 17β-estradiol (I) was used to produce a standard curve. Assay medium was used for blanks. The plate was incubated at 32° C and read on a microplate reader at 540 nm (Routledge and Sumpter, 1996). The assay has subsequently been applied to environmental samples such as sewage effluent fractions (Desbrow *et al.*, 1998) and used to examine the estrogenic potential of a range of manufactured chemicals (Routledge *et al.*, 1998a).

The yeast assay is extremely useful for the rapid determination of estrogenicity. However it has its limitations. Since it is a colorimetric technique, samples (pure compounds or environmental samples) possessing a high degree of colouration may mask the true estrogenicity. This may be particularly important for environmental samples which are often coloured.

The practicalities of the assay assume that the entire sample is taken up into the yeast medium and indeed into the yeast cell. Polar compounds with low log K_{ow} values would be expected to be easily taken up but there would be a degree of uncertainty for compounds with high log K_{ow} values as they may sorb to the plastic/glass micro plate (*cf* Zhou *et al.*, 1997).

1.1.3 Vitellogenin assay

Vitellogenin (Vg) is a phospholipoprotein, a precursor to egg protein, produced by all egg laying animals. 17β-estradiol (**I**), produced in the ovaries, stimulates the liver to produce vitellogenin in females but not in males. The liver excretes vitellogenin into the bloodstream where it is passed into the ovaries (EPA, 1997). As a result of xenoestrogen stimulation male animals can biosynthesise vitellogenin (Purdom *et al.*, 1994). The production of vitellogenin by male animals (such as the rainbow trout *Salmo gairdneri*) indicates estrogen stimulation and hence can be used as a biomarker for estrogen exposure during *in vitro* and *in vivo* studies (Herman and Kincaid, 1988; Purdom *et al.*, 1994; Palmer *et al.*, 1998). *In vitro* studies (Purdom *et al.*, 1994; Knudsen *et al.*, 1997,) and *in vivo* studies (Harris *et al.*, 1996; Herman and Kincaid, 1988; Christiansen *et al.*, 1998; Routledge *et al.*, 1998a; Tyler *et al.*, 1998) have all shown vitellogenin production in numerous fish. Laboratory studies may also be performed by culturing extracted hepatocytes (liver cells) of test animals, exposing them to samples and examining vitellogenin production (Jobling and Sumpter, 1993; Gagné and Blaise, 1998). Wild male fish can also be examined for plasma vitellogenin concentrations (Jobling *et al.*, 1998).

Since males do not produce vitellogenin, Vg synthesis in males is taken to indicate exposure to estrogens. Such exposure, however, could be due to the intake of non-estrogenic chemicals which then may stimulate estrogen production or lower androgen synthesis, allowing estrogen build up and hence vitellogenin production. Therefore the vitellogenin assay is probably best used in parallel with other estrogen assays.

1.1.4 Breast cancer cell assay (E-screen assay)

The E-screen uses the human breast cancer cell line, MCF-7, to determine estrogenicity. The cell proliferation is measured with or without 17β-estradiol (**I**) and the cell numbers determined (Soto *et al.*, 1995). Test compounds that produce cells greater than the control are estrogenic. Modifications of the E-screen have been used to analyse the estrogenic activity of STW effluents (Körner *et al.*, 1999).

1.1.5 Direct observation assays

There are a number of direct observation studies that may be used to indicate estrogen exposure including uterus weight increase and testicular growth in test species.

The increase in uterus weight of rodents (rodent uterotrophic assay) uses the fact that natural estrogens increase the size and weight of the uterus and uses the supposition that this could be artificially stimulated by administrating suspected xenoestrogens (Odum *et al.*, 1997; Ashby *et al.*, 1997). The results of the rodent uterotrophic assay have been shown to be comparable with the yeast assay. However, the ability of the uterotrophic assay to alter the structure of chemicals administered could increase estrogenicity leading to an increased result that would not be observed in the yeast

assay (Odum *et al.*, 1997). The uterotrophic assay may also be used to determine anti-estrogenicity which results in lowered 17β-estradiol (I) concentrations and a decrease in uterus weight (Odum *et al.*, 1997).

The observation of Odum *et al.* (1997) that estrogenicity can be increased by biotransformation could also lower the potential estrogenicity of a test compound. The determination of estrogen-indicating variables such as uterus weight should be conducted under stringent controls so as not to give a false positive result (Ashby *et al.*, 1997). The uterotrophic assay is expensive to run, labour intensive and uses a large number of test animals.

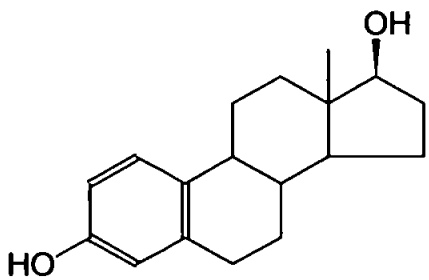
Immature male rainbow trout (*Oncorhynchus mykiss*) exposed to estrogens have been shown to reduce testicular growth (Jobling *et al.*, 1996). The gonadosomatic index, GSI, is the percentage size of the gonads compared with the total body weight (Jobling *et al.*, 1996). When known estrogens are administered there is a fall in GSI (compared with controls) indicating testicular growth inhibition. To confirm estrogenicity vitellogenin was observed in the blood plasma in one study (Jobling *et al.*, 1996).

Having reviewed the assays currently used to indicate estrogenicity in test chemicals, there now follows a brief summary of some of those chemicals over which there is currently concern. For a major review readers are referred to IEH (1995).

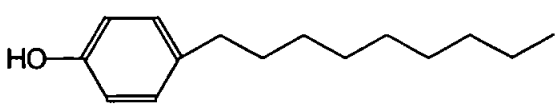
1.2 Types of estrogenic compounds

There are broadly speaking two classes of estrogenic compounds; natural estrogens, such as 17β-estradiol (I), and anthropogenic estrogen-mimicking compounds such as

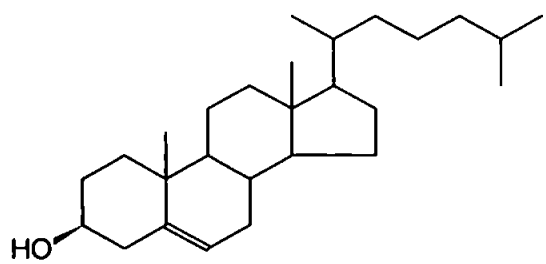
p-n-nonyl phenol (**II**). Natural estrogens can be considered as two groups, the mammalian estrogens and the phytoestrogens, of which the mammalian estrogens are far more potent.



I

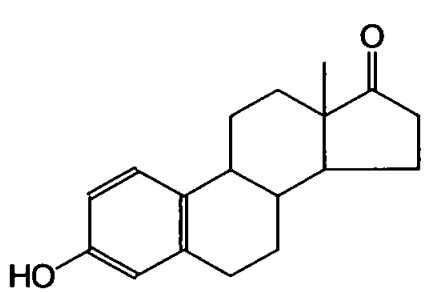


II

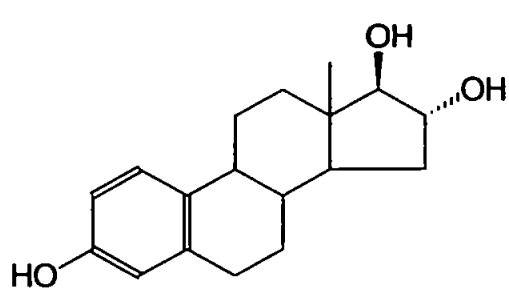


III

Female sex hormone production occurs primarily in the ovaries, from cholesterol (**III**), promoting sexual characteristics such as mammary gland enlargement. Estrogen production and excretion is continuous throughout the menstrual cycle but is maximised during ovulation. There are three main natural estrogens, 17β-estradiol (**I**), estrone (**IV**) and 16α,17β-estriol (**V**), all of which are A-ring aromatic steroids.

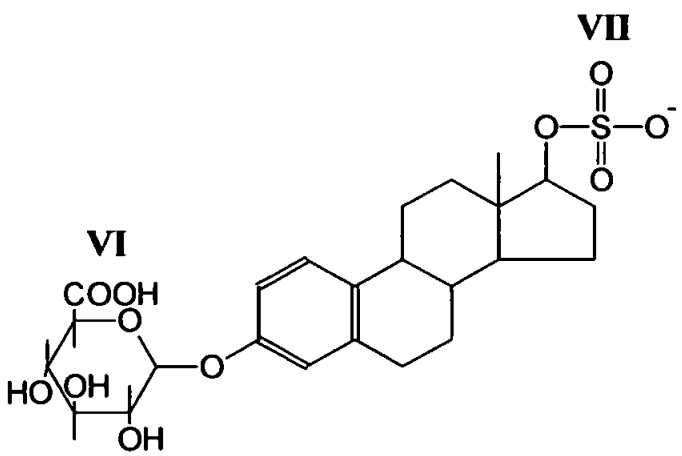


IV

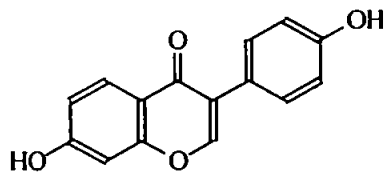


V

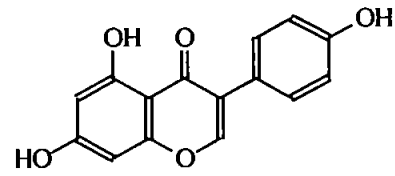
These are generally excreted from female humans in the urine (*ca* 14 μg day^{-1}) but up to 2.2 μg day^{-1} is excreted in the faeces (Adlercreutz *et al.*, 1986). Excretion of estrogens *via* urine involves the formation of the glucuronide (**VI**) or sulphate (**VII**) conjugates which are more water soluble than the parent steroids and have a lower estrogenicity (Desbrow *et al.*, 1998). In faeces, however, the steroids are usually in the more estrogenic unconjugated form (Desbrow *et al.*, 1998). There is speculation that the bioactivity of the conjugated estrogens in urine could be restored during sewage treatment by the hydrolysis of the conjugates by bacteria such as *Escherichia coli* (Desbrow *et al.*, 1998).



The so-called phytoestrogens, such as the isoflavones daidzein (**VIII**) and genistein (**IX**) are non-steroidal estrogens present in plants and hence occur in some common foods particularly in soya beans, and soya products, tofu, numerous vegetables and beverages such as beer (extensively reviewed by Bingham *et al.*, 1998; Reinli and Block, 1996; Rosenblum *et al.*, 1992).



VIII



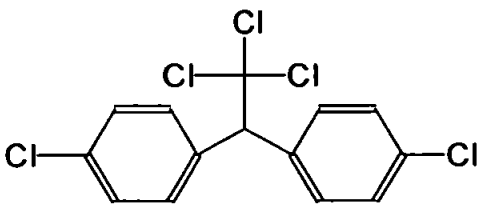
IX

Daidzein (**VIII**) has been shown to bind the estrogen receptor but seems to act as an anti-estrogen simply blocking the receptor and not triggering a biological response (Bingham *et al.*, 1998). Clover containing a derivative of **VIII** was believed to be responsible for mass infertility in sheep in Australia in the 1940s (Bingham *et al.*, 1998). **VIII** has been shown to be present in human urine and vegetarians excrete between 0.4-4.6 μ mol L^{-1} compared to 0.09 μ mol L^{-1} for cattle (Bannwart *et al.*, 1984). The phytoestrogens, isoflavonoids, have been shown to be estrogenic but with a potency 10^6 times lower than 17β-estradiol (**I**) (Markiewicz *et al.*, 1993). Concentrations of the female egg protein, vitellogenin (Vg) have been shown to be stimulated in the Siberian sturgeon (*Acipenser baeri*) when exposed to phytoestrogens (Pelissero *et al.*, 1991) (Table 1.2).

Compound	**Dose (mg g^{-1})**	**Vitellogenin produced (μg mL^{-1})**
17β-Estradiol (**I**)	0.0001	246 ± 97
Genistein (**IX**)	0.2	213 ± 56
Daidzein (**VIII**)	0.2	2 ± 0.06
Control	-	<0.1

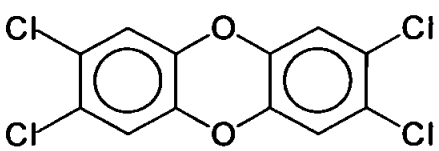
Table 1.2 Vitellogenin response to phytoestrogens and 17β-estradiol (**I**) in the Siberian sturgeon *Acipenser baeri* (Pelissero *et al.*, 1991).

The organochlorine pesticide 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) (**X**), at one time used world-wide as a powerful insecticide (Sharp, 1990), has been shown to cause egg shell thinning in a number of marine birds and has caused feminisation of the California gull (*Larus californicus*) due to ingestion of pesticides that bioaccumulate in fish lipids (Fry and Toone, 1981). Ingestion of a number of isomers of **X** into Californian gull eggs showed that *o,p'*-DDT caused feminisation (Fry and Toone, 1981). Also *p,p'*-DDE has been shown to act as an antagonist, binding to the androgen receptor in rats, resulting in feminisation (Kelce *et al.*, 1995).



X

Polychlorinated dibenzo-*p*-dioxins are by-products of PCB manufacture and occur in chlorine-containing bleach effluents from pulp and paper industries (IEH, 1995). Of the numerous isomers 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (**XI**) appears to be the most potent estrogen.

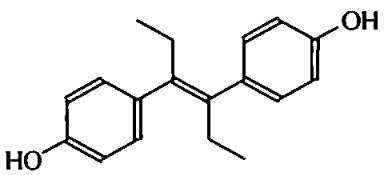


XI

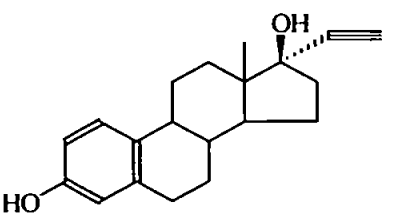
Female Rhesus monkeys (*Macaca mulatta*) exposed to **XI** at low concentrations showed that serum levels of 17β-estradiol (**I**) had been altered and that the animals were unable to breed (Barsotti *et al.*, 1979). In an *in vivo* bioassay **XI** was shown to

decrease hepatic and uterine weight compared to the weight increase observed when 17β-estradiol (**I**) was administered (IEH, 1995). This suggests that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (**XI**) acts as an anti-estrogen.

DES, diethylstilbestrol (**XII**), was used from the 1950s to 1970s as a means of preventing miscarriages in early pregnancy (IEH, 1995). There were alarming abnormalities in female offspring such as a rare vaginal cancer and disrupted menstrual cycles. Male offspring showed evidence of endocrine disruption with lowered sperm counts and testicular cancer. Such abnormalities resulted in **XII** being banned in early 1970s.



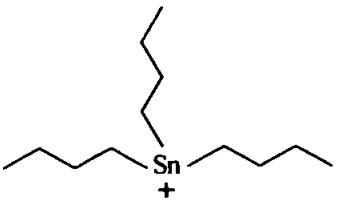
XII



XIII

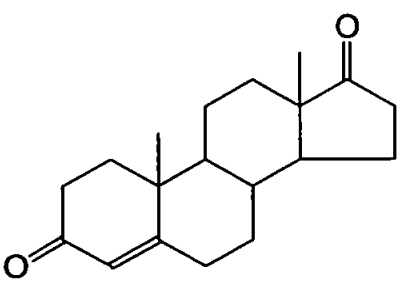
17α-ethynylestradiol (**XIII**), first synthesised over 60 years ago, is the principal estrogen in female oral contraceptives (Guengerich, 1990). As with 17β-estradiol (**I**) **XIII** is excreted in the urine in the conjugated form and is thought to be de-conjugated and re-activated by bacterial hydrolysis in STW (Desbrow *et al.*, 1998).

Tributyltin (TBT), as oxide or chloride (**XIV**), used in marine paints as an antifouling agent and wood preservative, has been shown to act as an androgen in the dogwelk (*Nucella lapillus*) causing masculinisation (Bryan *et al.*, 1986).

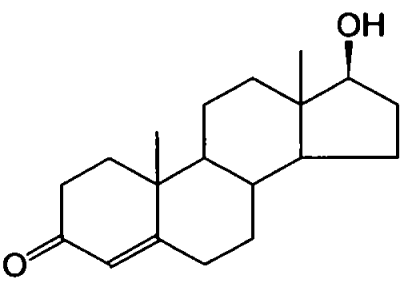


XIV

The female dogwelks showed significant concentrations of testosterone after exposure to TBT (**XIV**) (Spooner *et al.*, 1991). Laboratory studies confirmed that **XIV** blocks the aromatase enzyme used to convert androgens (androstenedione (**XV**) and testosterone (**XVI**)) into 17βestradiol (**I**) and estrone (**IV**) (Bettin *et al.*, 1996).

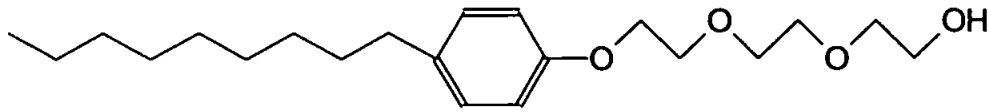


XV

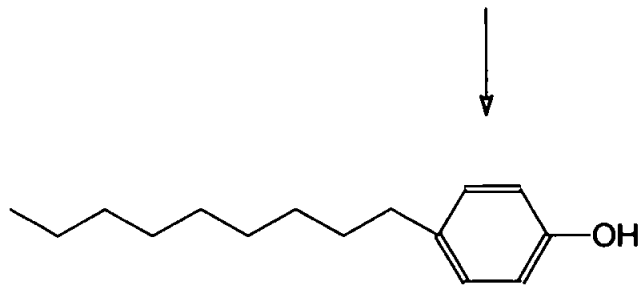


XVI

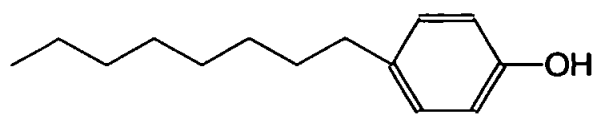
Alkylphenol ethoxylates (APEs) (*e.g.* nonylphenol triethoxylate (**XVIII**)) are a group of industrial nonionic surfactants used in detergents, paints and cosmetics. During microbial breakdown in sewage treatment works the APEs are biodegraded to smaller ethoxylate groups and eventually to alkylphenol (Giger *et al.*, 1987). The most commonly identified alkylphenols in sewage effluent are *p*-*n*-octylphenol (**XVII**) and *p*-*n*-nonylphenol (**II**) (Jobling *et al.*, 1995a).



Nonylphenol triethoxylate (**XVIII**)



II

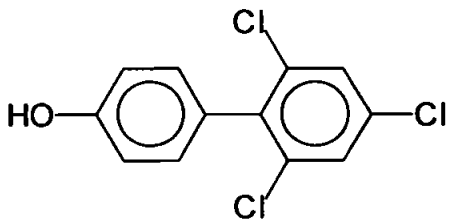


XVII

II has been identified in large amounts in sewage sludge and marine sediments (Wahlberg *et al.*, 1990; Marcomini *et al.*, 1990). Jobling *et al.* (1995a) determined that alkylphenols were estrogenic to immature male rainbow trout causing vitellogenin production and inhibition of testicular growth but 10,000 times less potent than 17β-estradiol (**I**).

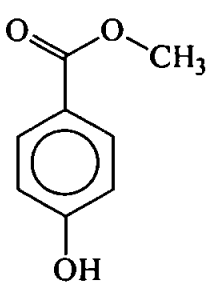
Polychlorobiphenyls (PCBs) had many uses and are persistent in the environment (IEH, 1999). For example, they were used in the past as flame-retardants and are also present in transformer oils. Although some of the 204 PCB congeners are estrogenic they are at least 10^6 times less active than 17β-estradiol (**I**) (IEH, 1995). Their estrogenicity appears to decrease with increased chlorination (IEH, 1995). The

hydroxylated PCB 4-hydroxy-2',4',6'-trichlorobiphenyl (**XIX**) binds strongly to the estrogen receptor and stimulates uterine weight increase in mice (Korach *et al.*, 1988).

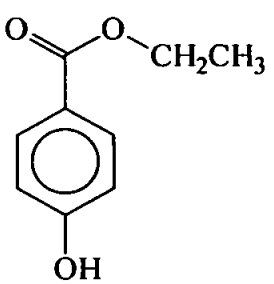


XIX

Routledge *et al.* (1998b) identified that the group of industrial chemicals collectively known as parabens (esters of *p*-hydroxybenzoic acid) show estrogenic activity. Parabens, of which methyl (**XX**), ethyl (**XXI**), propyl and butyl parabens are the most common, are used in numerous cosmetic applications such as shampoo and toothpaste



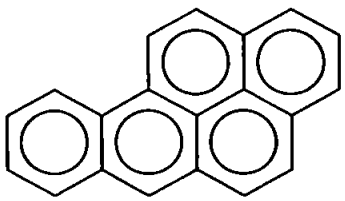
XX



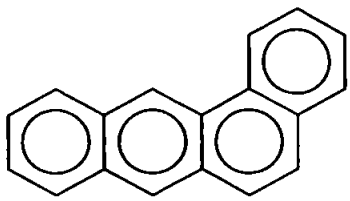
XXI

as preservatives. **XX** has been identified in vaginal secretions of female dogs in estrus and was shown to induce sexual arousal in male dogs (Goodwin *et al.*, 1979).

Polyaromatic hydrocarbons (PAHs) such as benzo(a)pyrene (**XXII**) and benz(a)anthracene (**XXIII**) have been examined for their estrogenic and anti-estrogenic properties due to some structural similarities to natural steroidal estrogens (reviewed by Santodonato, 1997)



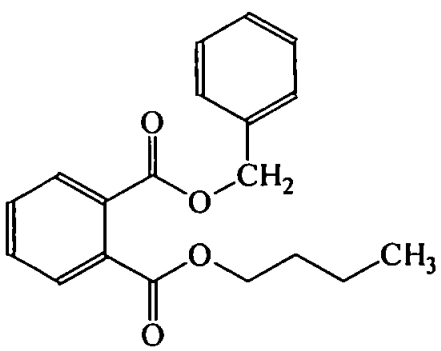
Benzo(a)pyrene (**XXII**)



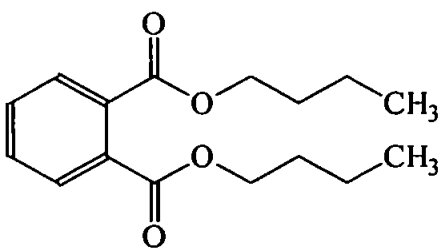
Benz(a)anthracene (**XXIII**)

Some PAHs are potent carcinogens in test animals but also show a weak estrogenic/anti-estrogenic potential (Santodonato, 1997).

Phthalate esters are a large group of man-made chemicals commonly found in plastics (plasticisers) such as PVC. As a result several phthalate esters are ubiquitous in the environment and are known to bioaccumulate in animal lipids (Jobling *et al.*, 1995b).



Butyl benzyl phthalate (**XXIV**)



Dibutyl phthalate (**XXV**)

Jobling *et al.* (1995b) examined common phthalate esters, **XXIV** and **XXV** and found them to exhibit a weak estrogenic activity (> 10^6 times less potent than 17β-estradiol (**I**)).

Thus a diverse number of organic chemicals have been suggested to have some 'estrogenic' activity but most active by far appear to be A-ring aromatic steroids such as 17β-estradiol (**I**), estrone (**IV**), 17α-ethynylestradiol (**XIII**) and 16α,17β-estriol (**V**).

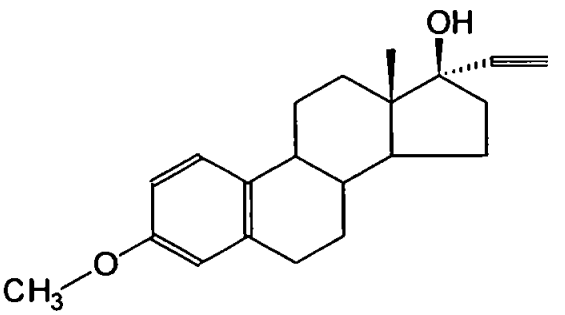
1.3 Recent studies of estrogenic compounds in treated wastewaters

Most research into the presence of estrogenic chemicals in wastewaters has been conducted since about 1980. However, a few earlier studies were made. For instance, Tabak and Bunch (1970) investigated the fate of natural and synthetic steroidal hormones in activated sludge and influent. The results indicated that the natural estrogens such as 17β-estradiol (**I**), estrone (**IV**), estriol (**V**) and the sterol cholesterol (**III**) were all present in sewage but that all were significantly biotransformed within 4-weeks (Table 1.3).

	Weeks incubated			
Steroid	1	2	3	4
	% Removed			
Cholesterol (**III**)	81	85	89	94
Estrone (**IV**)	70	79	85	89
17β-Estradiol (**I**)	68	78	85	89
Estriol (**V**)	66	74	81	86
17α-Ethynylestradiol (**XIII**)	55	61	70	79
Mestranol (**XXVI**)	22	30	46	59

Table 1.3 Average losses of steroids incubated in sewage over 4 weeks (Tabak and Bunch, 1970).

The synthetic estrogens, 17α-ethynylestradiol (**XIII**) and mestranol (**XXVI**) exhibited greater resistance to biodegradation than the natural steroids and appreciable proportions were still present after 4 weeks (Table 1.3).



Mestranol (**XXVI**)

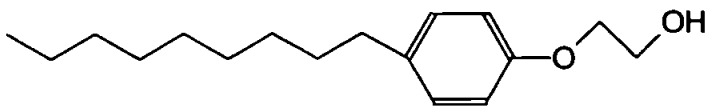
The discovery by anglers in the late 1980s of hermaphrodite roach in a STW lagoon catalysed considerable scientific research into the possible causes. The efficiency of STW employing primary and primary/secondary treatments, was investigated by Tabak *et al.* (1981). Samples of influent and effluent were taken from 14 STW over summer and winter periods, and these were extracted and analysed by TLC and GC. The results obtained were averaged to eliminate seasonal variability. The STWs employing secondary treatment were twice as efficient compared with STW employing primary treatment. The average influent concentration of natural estrogens ranged between 10-80 ng L^{-1} compared to 1.21 μg L^{-1} for the synthetic estrogens. Comparing concentrations in the influent and effluent the average biodegradation of the natural estrogens was between 33-75 % (w/w). The synthetic estrogens were again found to be far more resistant to metabolism with 5-25 % (**XXVI**) and 25-50 % (**XIII**) (w/w) being removed. Overall, the input of synthetic estrogens was far greater than that of natural estrogens. Furthermore, STWs employing primary treatment reduced the concentrations of synthetic estrogens in the effluent by 5-25 % compared with 20-40

% removal in secondary treatment plants. Shore *et al.* (1993) also examined the efficiency of STW and found that treatment removed 20-88 % estrogens (combined expression for 17β-estradiol (**I**) and estrone (**IV**)) and 60-77 % testosterone (**XVI**) from the raw sewage. Furthermore, estrogens were also discovered at low concentrations, in well (2.7 ng L^{-1} **I**) and irrigation water. The authors believed that 0.08 nmol L^{-1} (equivalent to 21.76 ng L^{-1} **I**) estrogens determined in lake drinking water would not have any serious effect on human health. These concentrations of combined estrogens were determined during an extreme drought where the water volume in the lake had been reduced by 83 % (v/v).

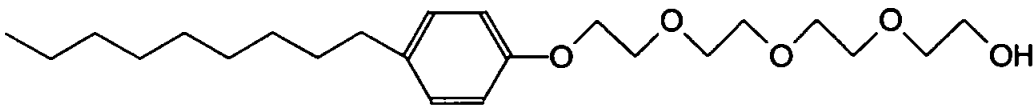
Ternes *et al.* (1999b) examined the breakdown of 17β-estradiol (**I**), estrone (**IV**), 17α-ethynylestradol (**XIII**) and **I** glucuronides by activated sludge in an aerobic batch experiment. The results show that 95% of **I** had been converted to **IV** within 3 h at high initial concentrations (1 μg mL^{-1} **I**). At lower concentrations (1 ng mL^{-1} **I**) **I** and **IV** were below detection limits after 5 h. 70 % **I** glucuronide had been converted to **IV** in ~25 h (**IV** was detected after 15 minutes). **XIII** was reduced but significant amounts remained after 50 h. 80 % of mestranol (**XXVI**) was biotransformed in 24 h. **XIII** was determined as the hydrolysis product but only 7% of **XXVI** could only be accounted for.

The alkylphenol-polyethoxylates (APEs) and intermediates, which are a group of non-ionic surfactants, have been shown to be present in sewage and rivers (Giger *et al.*, 1984; Marcomini *et al.*, 1990; Wahlberg *et al.*, 1990). Groundwaters around Cape Cod, Massachusetts have been shown to have significant APEs content around septic

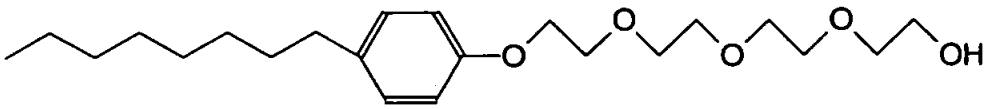
tanks due to leaching (Rudel *et al.*, 1998). Analysis of private drinking water wells has also identified nonylphenol monoethoxylate (**XXVII**), 4-nonylphenol tetraethoxylate (**XXVIII**) and octylphenol tetraethoxylate (**XXIX**) (Rudel *et al.*, 1998).



XXVII



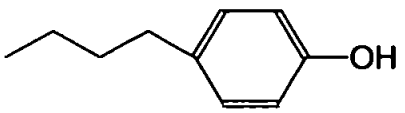
XXVIII



XXIX

During sewage treatment APEs have been shown to degrade to short chain APEs and eventually alkylphenols (AP) (Giger *et al.*, 1987). Jobling and Sumpter (1993) examined a number of AP derivatives to determine the estrogenic potential using a bioassay based on vitellogenin production in rainbow trout (*Oncorhynchus mykiss*). Vitellogenin (Vg) egg yolk protein is produced in the liver of female oviparous fish, amphibians and egg laying mammals as a direct response to 17β-estradiol (**I**). The exposure to exogenous estrogenic compounds in the female trout stimulates vitellogenin synthesis. The production of vitellogenin in male trout would indicate an exposure to estrogenic compounds. The hepatocytes from the livers of the male trout were cultured and exposed to a suite of AP as well as **I**. The secretion of vitellogenin proved that AP are estrogen mimics but with a potency of between 1 x 10^{-4} and

1 x 10^{-6} that of **I**. For 10 μM concentration the most potent estrogen mimics to induce vitellogenin were pure *n*-butylphenol (**XXX**) and *n*-octylphenol (**XVII**). Testicular growth in immature male trout was reduced by 50 % when exposed to 30 μg L^{-1} **XVII** (Jobling *et al.*, 1996).



XXX

A similar inhibition in testes development was obtained for 2 ng L^{-1} 17α-ethynylestradiol (**XIII**). AP compounds mimic estrogens by competing for the estradiol receptor displacing 17β-estradiol (**I**) (White *et al.*, 1994). This has been shown in trout and mice (White *et al.*, 1994).

The susceptibility of *Oncorhynchus mykiss* to estrogenic mimicking compounds was further utilised for *in situ* examination of STW effluent (Purdom *et al.*, 1994). Rainbow trout and carp (*Cyprinus carpio*) (both immature group) were kept in cages outside STW effluent outfalls. The experiment continued for up to 3 weeks, after which time the fish were killed, blood plasma extracted and the levels of vitellogenin determined. The results showed a marked increase in the vitellogenin concentration indicating the effluent contained estrogenic compounds. Preliminary laboratory studies, also on male trout, indicated that fish injected with 17α-ethynylestradiol (**XIII**) produced vitellogenin. The amount of **XIII** required to produce vitellogenin was far less than 17β-estradiol (**I**) and hence **XIII** was more potent than **I**. Harris *et al.* (1996) also examined the production of vitellogenin in male trout caged outside of STW effluents along the River Lea, U.K. The cages were placed close to the 5 effluent

outfalls and at known distances further down the river. The results showed that trout caged directly outside the effluent outfall had higher concentrations of plasma vitellogenin compared to trout caged further down stream. The tailing off of vitellogenin production at sites further away from the outfall was believed to be due to simple dilution effects. Control studies were performed in storage reservoirs. There was no statistical increase in vitellogenin concentrations. Jobling *et al.* (1998) examined wild roach (*Rutilus rutilus*) from rivers, lakes and canals. Four sample sites received no STW effluent, eight received effluent from one or more STW and one site was a control site. Intersexed fish occurred at all sites but occurrence was significantly higher in waters receiving treated effluent. Two rivers, the Nene and Aire had a 100 % incidence of intersex roach downstream of STW. The cause of intersexuality could not be distinguished between estrogens feminising males or androgens masculinising females, but was assumed to be the former due to the estrogenic content of STW effluent. Plasma vitellogenin concentrations were greatest in the female roach from all but the levels in control laboratory, and environmental sites were double that found in upstream/downstream sites. This could suggest an inhibition of the estrogen receptor or the action of androgens defeminising female fish. For the intersexed and male fish the vitellogenin was greatest downstream of STW effluent outfalls.

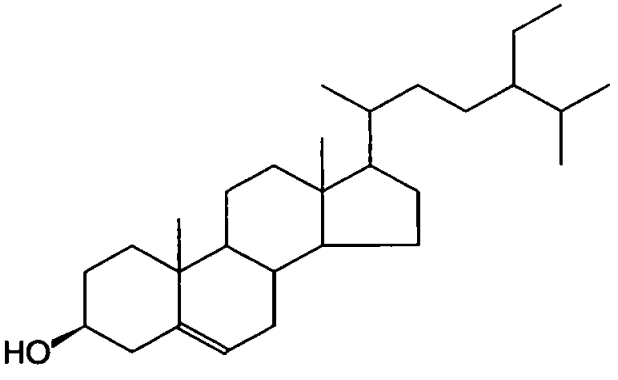
The compounds causing this vitellogenin production in male trout had not, at the inception of the present study, been fully defined. Subsequent to this study Desbrow *et al.* (1998) examined sewage effluents using a novel extraction and toxin identification procedure. Sewage effluent samples, (20 L), were taken directly from STW outfalls and extracted by passing through octadecylsilane (C_{18}) solid phase extraction (SPE) cartridges. The resulting retained organic matter was eluted from the

cartridges and subsequently fractionated by HPLC. Small aliquots of each fraction were screened by the Yeast Assay (genetically altered yeast where the human estrogen receptor gene has been incorporated into the main genome (Glaxo Welcome plc)) for estrogenic activity. Those fractions giving a positive assay were analysed by GC-MS. Three steroid estrogens were identified, 17β-estradiol (**I**) and estrone (**IV**) and the synthetic 17α-ethynylestradiol (**XIII**). The concentrations ranged between 0.2 to 7 ng L^{-1}. **XIII** was generally not present in effluent which had been processed beyond primary treatment. The concentrations of **I** and **IV** were also greater in STW plants that generally only employed primary treatment. Concentrations of **IV** were as high as 47 ng L^{-1} (ranging between 6.3-47 ng L^{-1}). More recently concentrations of **I** ranging between 0.7-12 ng L^{-1} have been reported in STW effluents from the Netherlands (Belfroid *et al.*, 1999). Ternes *et al.* (1999a) found concentrations of **IV** in German STW effluents as high as 70 ng L^{-1} (median 9 ng L^{-1}), concentrations of **I** were <1 ng L^{-1}. Concentrations of **I** and **IV** as high as 48 ng L^{-1} and 64 ng L^{-1} respectively were determined in STW effluents in Canada (Ternes *et al.*, 1999a).

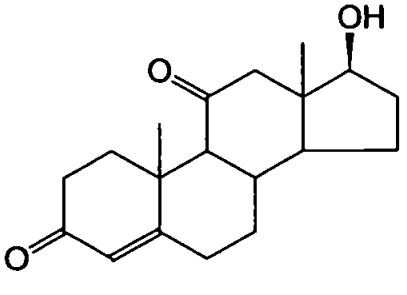
The concentrations of natural estrogens found in STW effluents were used in laboratory studies on trout and roach to determine whether such concentrations were sufficient to cause a vitellogenin response (Routledge *et al.*, 1998a). The results indicated that 10 ng L^{-1} 17β-estradiol (**I**) and 25 ng L^{-1} estrone (**IV**) was enough to cause a significant vitellogenin response in trout. The concentrations of vitellogenin produced in plasma after treatment with either 25 ng L^{-1} of **I** or **IV** were about 1 μg mL^{-1}. The combination effect of **I** plus **IV** was also examined on male trout to assess the possibility of a synergistic response. The addition of 25 ng L^{-1} of **I** plus **IV**

produced a dramatic vitellogenin response of 17.4 ± 6 mg mL^{-1}. This compares to a maximum vitellogenin production in female trout of 50 mg mL^{-1}.

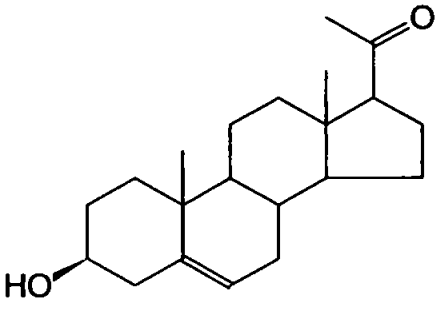
The natural plant sterol, β-sitosterol (**XXXI**) has been shown, at concentrations between 20 to 100 μg g^{-1}, to alter hormonal steroids in blood plasma when injected directly into male and female goldfish (MacLatchy and Van der Kraak, 1995). There were significant reductions in plasma testosterone (**XVI**) and 11-ketotestosterone (**XXXII**) concentrations in male fish and plasma **XVI** and 17β-estradiol (**I**) concentrations in female fish.



XXXI



XXXII



XXXIII

The reasons are unclear but it has been suggested that **XXXI** may compete with cholesterol (**III**) for enzymes required to convert **III** into prognenolone (**XXXIII**) (side chain cleavage). This would decrease the amounts of estrogens/androgens in the blood plasma.

β-sitosterol (**XXXI**) has been identified in sewage sludge and in the liquid and solid fractions of influents and effluents (Nguyen *et al.*, 1995; Quéméneur and Marty, 1994). The total concentration (liquids and solids) of **XXXI** in sewage influent has been calculated at between 18 to 483 μg L^{-1} (Quéméneur and Marty, 1994). In the effluent between 10-120 μg L^{-1} has been determined (Quéméneur and Marty, 1994). Much higher concentrations of **XXXI**, between 280 to 1200 μg L^{-1}, have been reported in paper mill effluent (MacLatchy and Van der Kraak, 1995).

Although the input of steroid estrogens into the environment is not limited to sewage effluent and human activity these are likely to represent by far the largest inputs. Domesticated animals such as cattle, horses and chicken are known to excrete large amounts of steroids, including estrogens, directly into the environment (Shore *et al.*, 1993; Leeming *et al.*, 1996). Poultry litter applied to the land has been shown to leach 17β-estradiol (**I**) to the land (Nichols *et al.*, 1997).

1.4 Methods for the determination of steroids from complex mixtures

The quantification of steroidal compounds in complex biological mixtures such as serum, urine, hair, tissue and faecal samples has generally been achieved by chromatographic based techniques such as HPLC, TLC and GC. There are other

techniques such as immunoassays that have become increasingly popular (Townsend, 1995; review of analytical techniques used in the study of steroids). Prior to analysis most biological samples must first undergo preliminary extraction to remove the bulk of unwanted material. For liquid samples such as blood, plasma, urine and STW effluents SPE has been successfully employed in pre-concentrating analytes (Hooijerink *et al.*, 1994; Hrdlicka *et al.*, 1997; Lee and Peart, 1998; Rule and Henion, 1999). The extraction of solid samples such as faeces and sludge usually require a solvent extraction step or supercritical fluid extraction prior to SPE (Schoene *et al.*, 1994; Nguyen *et al.*, 1995; Hamoir *et al.*, 1998). After pre-concentration and bulk removal of unwanted material the sample can be analysed.

TLC has been used to great effect in the separation and quantification of steroids. The main application of TLC in the analysis of steroids has been to examine bulk steroids, generally by the pharmaceutical industry, to assess for purity (Horvath *et al.*, 1997). The use of TLC enables a fast, cheap and semiquantitative analysis. Tabak *et al.* (1981) used TLC to identify numerous hormones in sewage influent and sludge.

Liquid chromatography (LC) application in the analysis of steroids has been in the refining of a sample by fractionation prior to quantitative analysis. Desbrow *et al.* (1997) used a HPLC fractionation procedure to cleanup and pre-concentrate estrogenic steroids from sewage effluents prior to GC-MS analysis. Hooijerink *et al.* (1994) examined plasma from cattle for anabolic steroids content and used HPLC to remove unwanted material before GC-MS analysis. The coupling of LC systems to a mass spectrometer has, however, enabled quantitative sample analysis (*e.g.* Guillemette and Belanger, 1995). Murry *et al.* (1996) used LC-MS (electrospray ionisation mass

spectrometer) to analyse urine samples for sulphate conjugated estrogens such as 16α,17β-estriol 3-sulphate and estrone 3-sulphate. Before LC-MS, GC-MS was the preferred analytical tool to study sulphate conjugated steroids. Many conjugated steroids are, however, thermally unstable and thus analysis by GC-MS requires an initial hydrolysis step to remove the conjugate (Belfroid *et al.*, 1999; used the enzyme β-glucuronidase as deglucuronidation agent). For LC-MS analysis of conjugated steroids no sample preparation, prior to analysis, is required. Murry *et al.* (1996) reported that the limit of detection (LOD) for 16α,17β-estriol 3-sulphate and estrone 3-sulphate was *ca* 100 pg per steroid on column. Furthermore, the use of LC-MS can also increase sample throughput when compared with GC-MS. Zang and Henion (1999) used SPE prior to LC-MS (tandem triple-quadrupole mass spectrometer with turbo ion spray source in negative ionisation mode) in the analysis of estrogen sulphates from urine. The authors reported that 192 samples could be analysed per day and with a LOD of *ca* 0.2 ng mL^{-1}.

Gas chromatography (GC) has been the preferred quantitative analytical tool in the analysis of steroids. Separation, especially by capillary GC, is highly sensitive and coupled with either a mass spectrometer (MS), electron capture detector (ECD) or a flame ionisation detector (FID) allows detection limits of sub pg levels on column (GC-MS analysis of biological samples extensively reviewed by Wolthers and Kraan, 1999). Schoene *et al.* (1994) studied the metabolism of 17α-methyltestosterone in horses using GC-MS and compared electron ionisation (EI) and chemical ionisation (CI) techniques. The use of CI mode allowed more sensitive determination of the molecular ion ($M^{+\cdot}$) of metabolites (as the TMS ether derivatives) produced compared

with EI. When comparing the LOD between FID and ECD on a suite of free sterols, Baiocchi *et al.* (1996) identified that on average the LOD of the FID was *ca* 50 ng on column compared with *ca* 1 – 5 ng on column for the ECD. The authors reasoned the difference by the presence of keto and hydroxy groups acting as electron absorbers. Although less sensitive Munoz *et al.* (1997) used GC-FID to great effect as an initial examination of petroleum polluted soils for sterene and mono aromatic steroid analysis. The more sensitive GC-MS was used to confirm pollutants present.

The GC analysis of hydroxysteroids is generally not as the free alcohol but as a derivative such as trimethylsilyl esters (Nguyen *et al.*, 1995). Derivatisation helps to increase sensitivity by decreasing the polar nature of the hydroxy group. Kelly (2000) showed that ion trap GC-MS analysis of steroid estrogens from wastewater samples, as the *tert*-butyldimethylsilyl derivatives, could be detected at *ca* 1 ng L^{-1}. For greater sensitivity and selectivity GC-MS/MS (ion trap mass spectrometer) of estrogenic steroids can increase the LOD to *ca* 0.1 ng L^{-1} (Belfroid *et al.*, 1999).

The use of immunoassay methods in the analysis of steroids has enabled researchers to determine specific steroids (at very low levels) in complex biological matrix. The principles of immunoasssays are based on the production of an antibody as a result of immune responses to a specific antigen added (*e.g.* a specific steroid). The antibody produced, which will be antigen specific, can, therefore, be used to signal the presence of the same antigen in a test sample. The use of immunoassays is relatively cheap, as well as being analyte specific and extremely sensitive. However, the use of immunoassays for steroids can suffer from cross reactivity of closely related analytes with the antibody thereby influencing the sensitivity and overall concentrations

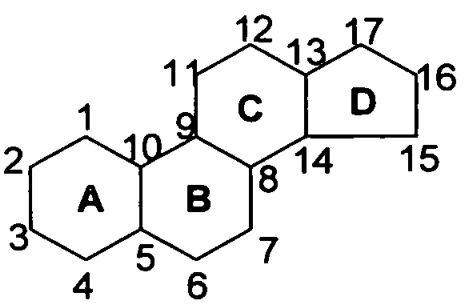
(Wolthers and Kraan, 1999). Townsend (1995) reviewed many immunoassay techniques used in the analysis of steroids. A brief description of three standard techniques used is outlined below.

Male trout respond to environmental estrogens such as 17β-estradiol (**I**) by synthesising the female egg protein precursor, vitellogenin, in the liver. For the determination of vitellogenin concentrations in plasma a radioimmunoassay (RIA) was developed with a LOD of *ca* 0.1 μg mL^{-1} (Tyler and Sumpter, 1990). RIA are classed as ligand-binding assays, where an antigen of interest (*i.e.* vitellogenin) binds to a unique antibody. To determine specific antigen concentrations the unused antibodies are calculated by the addition of a radiolabelled antigen (*e.g.* ^{3}H vitellogenin) which in turn complex. The radiolabelled antigen/antibody complex is washed free of any non-bonded radiolabelled antigen and the radioactivity of the complex determined by standard techniques. Many authors have used the RIA for vitellogenin to assess the exposure of numerous wide male fish species to estrogenic chemical in the environment (Purdom *et al.*, 1994; Harris *et al.*, 1996; Jobling *et al.*, 1998; Routledge *et al.*, 1998a). The RIA technique is, however, dependent on the stability of the radiolabelled compound used and can also be expensive to dispose of once used (Kohen *et al.*, 1986). In an attempt to overcome these disadvantages chemiluminescent immunoassays (CIA) have been used. The radioactive antigen in RIA is replaced with an antigen such as a steroid or steroid glucuronide which has a chemiluminescent molecule such as isoluminol bonded to it. The same basic procedure used for RIA can be used for CIA but detection is by a luminometer (Kohen *et al.*, 1986). Furthermore, fluorescence techniques such as dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) have been applied to the detection of steroids such as

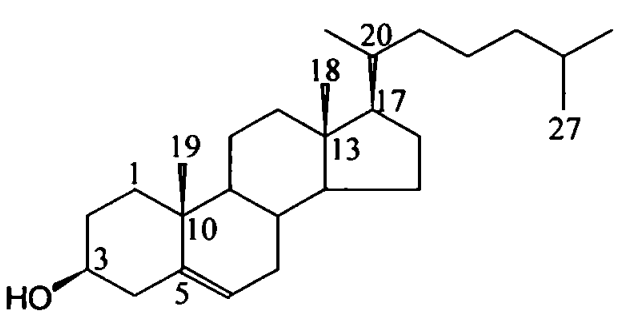
testosterone (**XVI**), 17β-estradiol (**I**) and progesterone (**XXXXVI**) with LOD ranging between 10 – 314 pg mL^{-1} (Elliot *et al.*,1995). Detection was by spectrofluorimetry.

1.5 Cholesterol biochemistry

Cholesterol (**III**) is the most abundant steroid in the body, occurring either as the free molecule (in cells and tissues) or bound to fatty acids in the form of cholesteryl esters (in blood). The cholesterol molecule is comprised of a 4 ring nucleus (cyclopentanoperhydrophenanthrene) to which are assigned the letters A, B, C and D with the carbons in the ring system numbered as shown below:



The A/B, B/C and C/D ring linkages are in the *trans* conformations. The methyl groups in positions 10 and 13, the alkyl group at C-17 and the hydroxyl group at position 4 are orientated in the β position (*i.e.* out of the plane as drawn).



III

1.5.1 Biosynthesis of cholesterol

In mammalian systems, cholesterol (**III**) can either be produced synthetically *in vivo* or ingested as a result of diet. The major production regions for **III** synthesis are the liver and intestines.

In mammals cholesterol (**III**) is ultimately derived from acetyl CoA (**XXXIV**) but **III** is usually considered to be made up of 6 isoprenoid units with the loss of 3 carbon atoms. The first step in the production of isoprene is the reaction of two **XXXIV** units, catalysed by thiolase enzyme, producing acetoacetyl CoA (**XXXV**). Acetyl CoA (**XXXIV**) and **XXXV**, under the influence of hydroymethylglutaryl-CoA synthetase, form 3-hydroxy-3-methyl-glutaryl-CoA (**XXXVI**). The enzyme reductase reduces **XXXVI** to mevalonate (**XXXVII**). **XXXVII** is converted to isopentenyl biphosphate (**XXXVIII**) *via* 2 intermediates (not shown) and 3 ATP. **XXXVIII** undergoes isomerisation to form dimethylallyl biphosphate (**XXXIX**), the starting material for the production of squalene (**XXXX**). **XXXVIII** and **XXXIX** undergo a condensation reaction to produce the C_{10} unit geranyl biphosphate (**XXXXI**). **XXXXI** and **XXXVIII** undergo further condensation to produce the C_{15} unit farnesyl biphosphate (**XXXXII**). The final step in the production of **XXXX** is the condensation reaction between two **XXXXII** molecules to form **XXXX**. **XXXX** is enzymatically oxidised producing squalene 2,3-epoxide (**XXXXIII**). Depending on the biological system, **XXXXIII** goes on to produce either lanosterol (mammals and fungi) or cycloartenol (plants and algae). **XXXXIV** undergoes demethylation at positions 14 and 4 (this order is specific for mammals), reduction of the double bond at position 24(25) and isomerisation of the double bond at position 8(9) to position 5(6), *via* a $\Delta^{5,7}$ sterol

intermediate, to produce cholesterol (**III**) (Figure1.1) (Benveniste, 1986; Stryer, 1995).

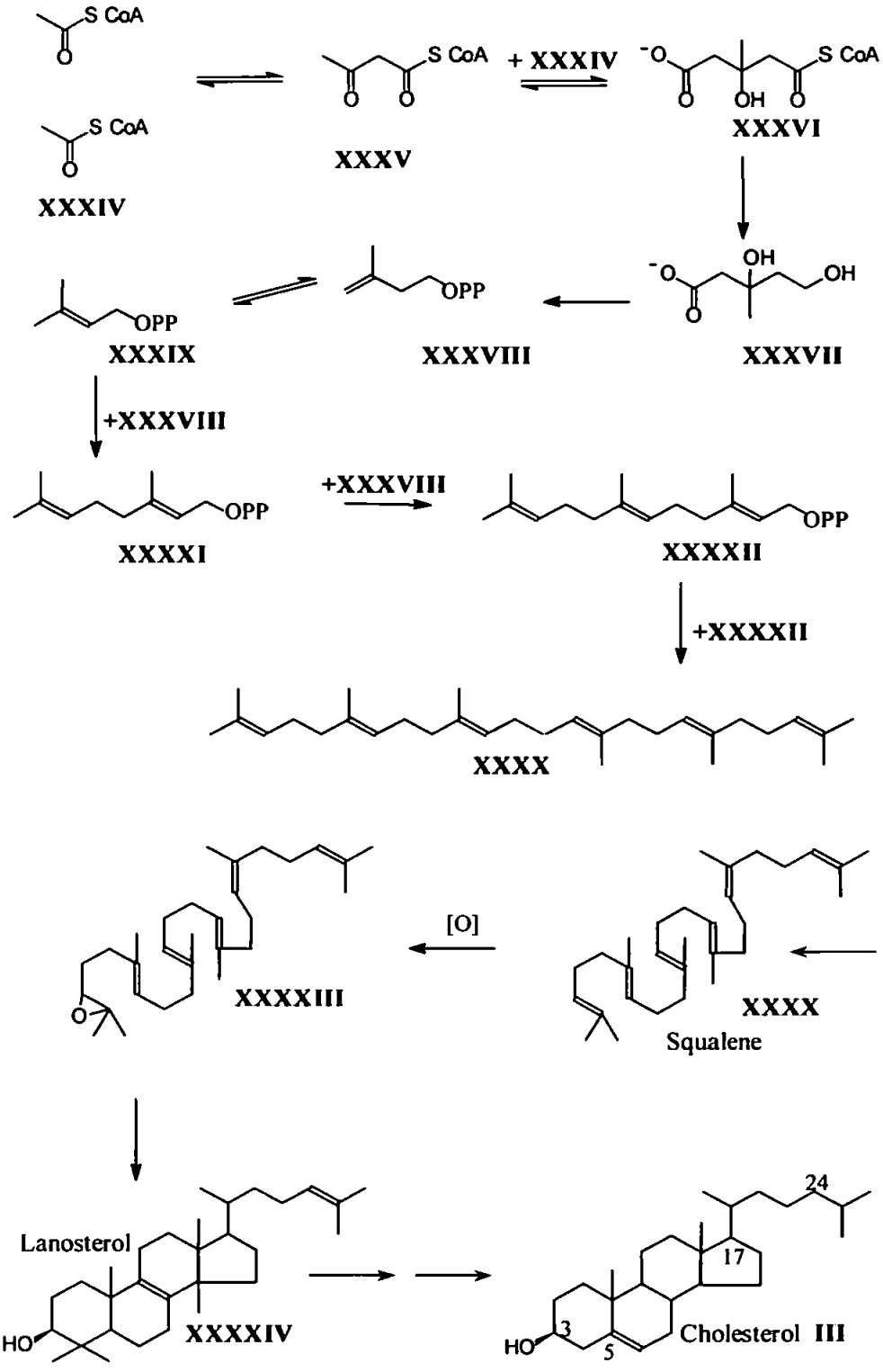


Figure 1.1 The biosynthesis of cholesterol in mammalian systems (PP = biphosphate). For simplicity enzymes are omitted.

1.5.2 Mammalian steroid hormone synthesis from cholesterol

The first step in the biosynthesis of steroid hormones is the cleavage of the alkyl chain at position 20. The cleavage occurs *via* the P450 cytochrome where hydroxylation occurs at positions 20 and 22 producing 20α,22β-dihydroxycholesterol (**XXXXV**) Figure 1.2. The bond between the two hydroxy groups is cleaved by the enzyme desmolase producing pregnenolone (**XXXIII**). The $\Delta^{5(6)}$ double bond is isomerised to position 4 and the hydroxy group at position 3 is oxidised to a keto group forming progesterone (**XXXXVI**). The acetyl group at position 17 is removed by hydroxylation (17α-hydroxyprogesterone, (**XXXXVII**)) followed by oxidation to a keto group yielding androstenedione (**XV**) and acetone. **XV** can either be used to synthesise testosterone (**XVI**) *via* the reduction of the keto group at position 17, or to form **IV** (estrone) by the demethylation of the methyl group at position 19, resulting in the aromatisation of the A-ring and reduction of the keto group at position 3. 17β-estradiol (**I**) is formed by the demethylation, aromatisation and reduction (as with **IV**) of **XVI** (Figure 1.2) (Benveniste, 1986; Stryer, 1995).

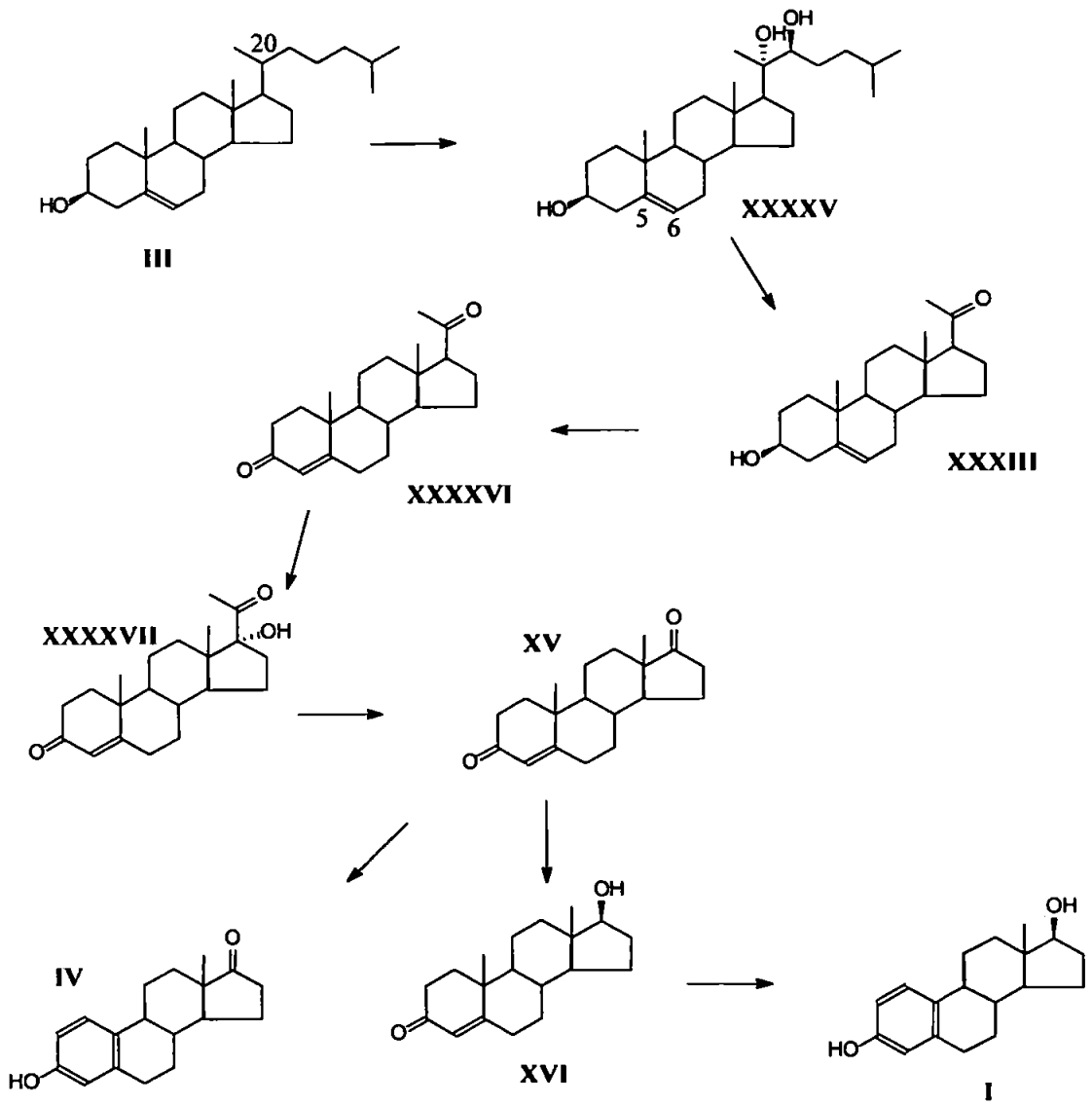
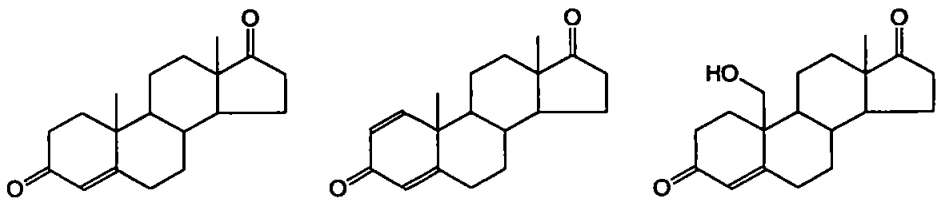


Figure 1.2 Mammalian biosynthesis of 17β-estradiol (**I**), estrone (**IV**) and testosterone (**XVI**) from cholesterol (**III**).

The formation of 17β-estradiol (**I**) and estrone (**IV**) from testosterone (**XVI**) was also shown to occur from androst-1,4-dien-3,17-dione (**L** *cf.* **XV**) but more rapidly from 19-hydroxy-androst-4-en-3,17-dione (**LI**) (Fieser and Fieser, 1959).



XXXXIX **L** **LI**

Aromatisation of testosterone (**XVI**) to 17β-estradiol (**I**) is catalysed by a cytochrome $P450_{arom}$ enzyme belonging to the CYP19 subfamily (Devlin, 1997). Firstly **XVI** undergoes enolisation to form androst-2,4-dien-3,17β-diol (**LII**). The angular methyl at position 19 then undergoes hydroxylation to form androst-2,4-dien-3,17β,19-triol (**LIII**) followed by androst-2,4-dien-3,17β,19,19-tetrol (**LIV**). **LIV** undergoes dehydration to form androst-2,4-dien-3,17β,-diol-19-one (**LV**). The final stages are the incorporation of a $Fe^{3+}OOH$ group to the 19 methyl (**LVI**). Subsequent cleavage of the 19-methyl results in aromatisation of the A-ring producing 17β-estradiol (**I**) (Figure 1.3) (Devlin, 1997).

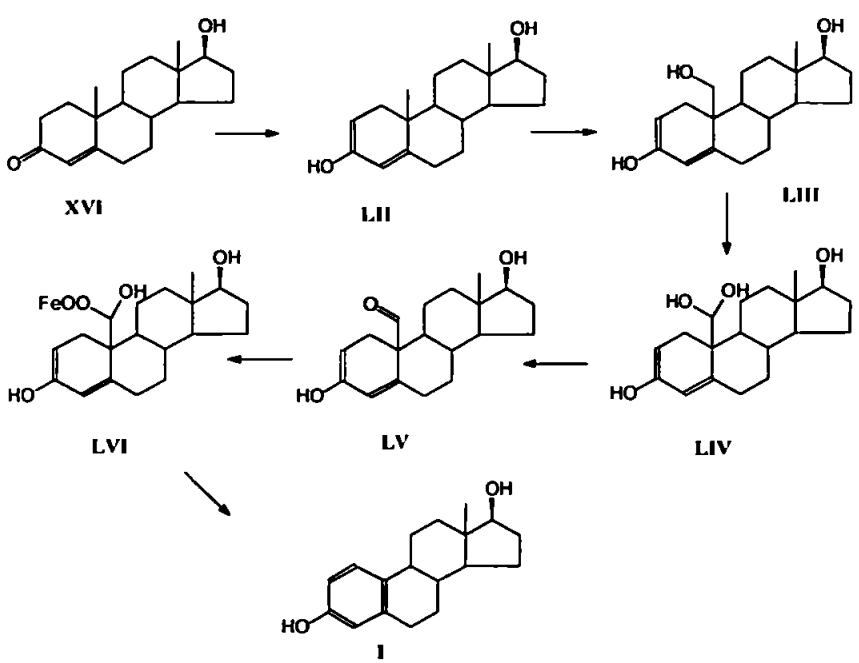


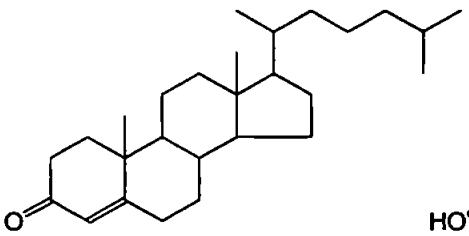
Figure 1.3 The mechanism for 17β-estradiol (**I**) biosynthesis from testosterone (**XVI**).

1.5.3 Metabolism of cholesterol and derivatives by micro-organisms

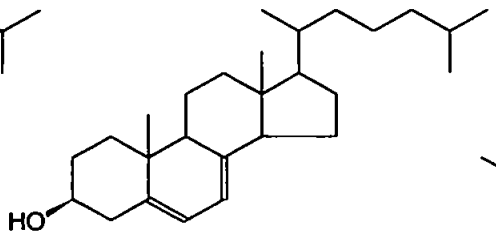
Whilst the above studies show that knowledge of mammalian cholesterol metabolism is quite advanced much less is known about the metabolism of cholesterol by microorganisms. Inputs of cholesterol (**III**) to the environment are numerous generally result from both mammalian excretion of **III** and releases of steroids from plants by microbial degradation of plant material. Biotransformations of **III** may then occur where the residence time is sufficient to allow microbial colonies to establish (in sewage treatment works, in soils including soil that receive sewage sludge and in marine, riverine and lacustrine sediments).

In order to study the biotransformation products of cholesterol (**III**) metabolism, culture experiments with single strains of microbes have been used most often in which micro-organisms cultured in the laboratory are grown with **III** as the sole organic carbon source. One disadvantage of this approach is the lack of naturally competing organisms that may have co-metabolic effects. Nonetheless a great deal has been learned from such studies most of which were conducted *ca* 30-50 years ago.

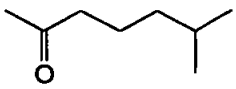
Harvath and Kramli, (1947) cultured *Azotobacter* with cholesterol (**III**). The main metabolite was cholest-4-en-3-one (**LVII**). However, 7-dehydrocholesterol (**LVIII**) and methyl heptanone (**LIX**) were also detected.



LVII

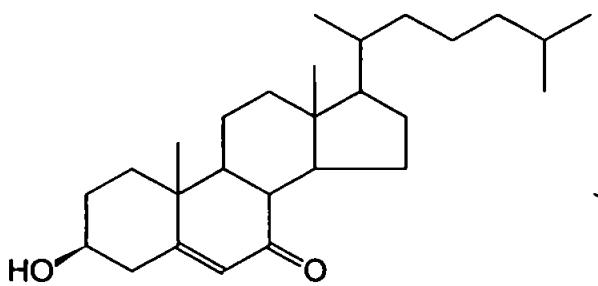


LVIII

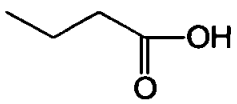


LIX

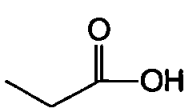
Later work provided the first reported microbial hydroxylation of a steroid with the isolation of 7-ketocholesterol (**LX**), assumed to form *via* 7-dehydrocholesterol, when *Proactinomyces roseus* was allowed to biodegrade cholesterol (**III**) (Harvath and Kramli, 1948, 1949).



LX



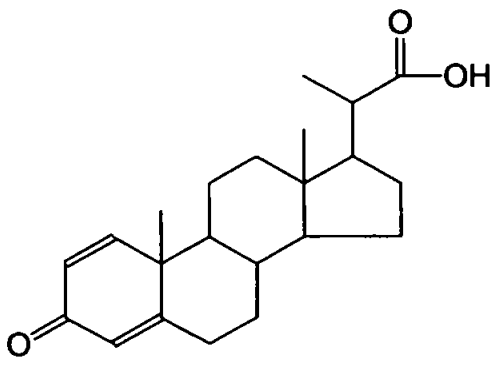
LXI



LXII

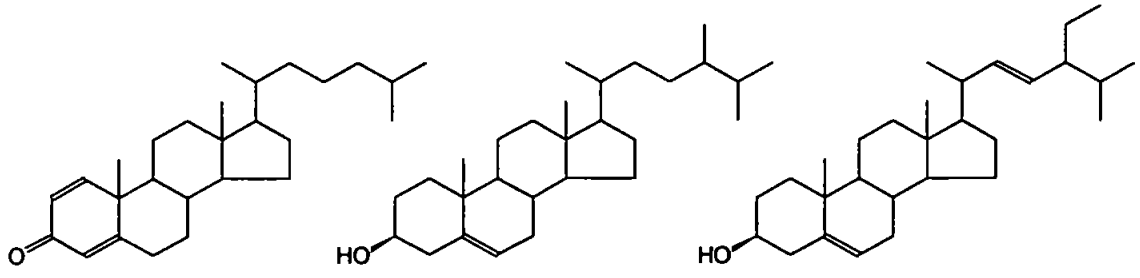
There has been a considerable amount of research into the microbial metabolism of the alkyl side chain. Sih *et al.*, (1967b) examined the breakdown of cholesterol (**III**) using 26,27-^{14}C radiolabelled **III** incubated with the bacterium *Norcardia restrictus*. *N. restrictus* metabolised the side chain producing 2 moles of propanoic acid (**LXI**), 1 mole of acetic acid (**LXII**) and 1 mole 17-keto steroid from 1 mole of **III**. 1 mole of **LXI** was found to contain both ^{14}C atoms indicating that a C24-25 cleavage had occurred. The non-labelled **LXII** and **LXI** were the result of C22-23 and C17-20 cleavages respectively. Before the removal of the final **LXI** (C17-20 cleavage) a C22

acid intermediate, 3-oxobisnorchola-1,4-dien-22-oic acid (**LXIII**), was formed (Sih *et al.*, 1967a).



LXIII

The bacterium, *Norcardia corallina*, was also shown later to produce **LXIII** from cholesterol (**III**) (Arima *et al.*, 1978). The side chain cleavage by *N. restrictus* is unlike that which occurs in mammalian systems in the production of hormonal steroids (Figure 1.2,). Nagasawa *et al.* (1969) also examined the cleavage of the side chain of **III** by *Arthrobacter simplex* and found that the initial metabolite was cholest-4-en-3-one (**LVII**). Further incubation yielded cholesta-1,4-dien-3-one (**LXIV**) and the side chain cleaved androst-4-en-3,17-dione (**XXXXIX**) and androst-4-en-3,17-dione (**L**). Similar sterols, campesterol (**LXV**), **LVIII**, **XXXI** and stigmasterol (**LXVI**), all produced androst-4-en-3,17-dione (**L**) in varying amounts.

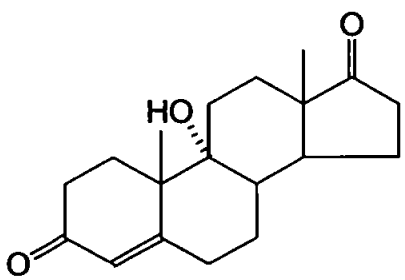


LXIV **LXV** **LXVI**

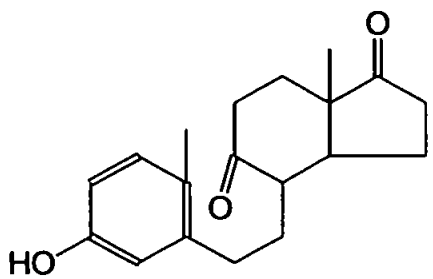
There is limited evidence on the cleavage of the alkyl side chain of sterols in the environment. Avsejs *et al.* (1998) extracted and analysed samples of peat and found a

high androstane content. The androstane compounds identified were not added directly to the peat from the dominant flora species (*Sphagnum* moss) but more likely were oxidation products of β-sitosterol (**XXXI**) present in the moss at high concentrations. Taylor *et al.* (1981) examined cholesterol (**III**) metabolism in anaerobic microbial enriched marine sediments and identified androst-4-en-3,17-dione (**XXXXIX**) among the metabolites produced.

XXXXIX incubated with *N. restrictus* has been shown to produce 9α-hydroxyandrost-4-en-3,17-dione (**LXVII**) and 3-hydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (**LXVIII**) (Dobson and Muir, 1961b). Dobson and Muir (1961a) also showed that the bacteria *Pseudomonas* and *Arthrobacter* metabolised **XXXXIX** to produce **LXVIII**.



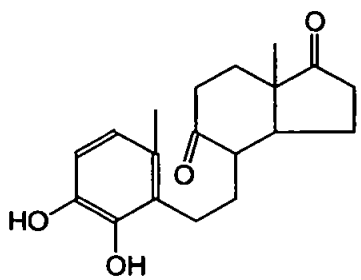
LXVII



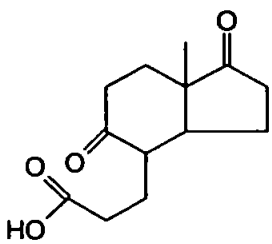
LXVIII

The bacterium *A. simplex* also metabolised **L** to yield **LXVIII** (Nagasawa *et al.*, 1969). The production of **LXVIII** requires the aromatisation of the A-ring and the cleavage of the B-ring. The order in which micro-organisms produce **LXVIII** is important as estrone (**IV**) might be an important intermediate prior to B-ring cleavage. The insertion of a hydroxyl in position 9 into **L** followed by oxidation to a keto group suggests B-ring cleavage before A-ring aromatisation (Nagasawa *et al.*, 1969). Furthermore **IV** incubated with *N. restrictus* did not undergo metabolism and hence would be an end-product rather than an intermediate (Sih and Wang, 1965c). 3-

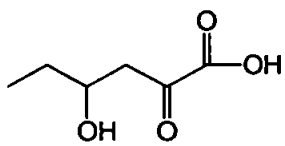
hydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (**LXVIII**) can undergo further biodegradation, by *N. restrictus*, and produces 3,4-dihydroxy-9,10-secoandrosta-1,3,5-trien-9,17-dione (**LXIX**) (Sih *et al.*, 1965a). The metabolism of **LXIX** by *N. restrictus* produced oxidative cleavage of the A-ring (C4-5) to 3aα-H-4α-[3'-propionic acid]-7aβ-methyl-hexahydro-1,5-indandione (**LXX**) and 2-oxo-4-hydroxycaproic acid (**LXXI**) (Sih *et al.*, 1965b).



LXIX

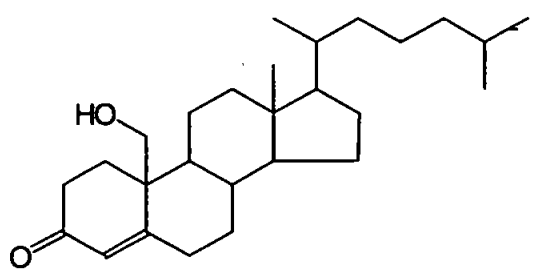


LXX

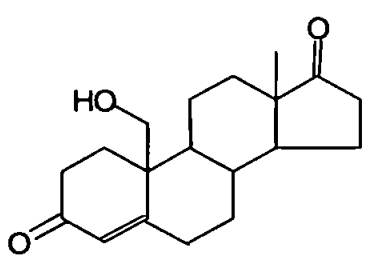


LXXI

Sih and Wang (1965c) incubated 19-hydroxycholest-4-en-3-one (**LXXIII**) with *N.restrictus* and obtained estrone (**IV**) (8 % yield). They discovered that the amount of **IV** obtained accumulated as *N. restrictus* was shown not to metabolise **IV**. Indeed the incubation procedure was repeated for CSD-10, an organism isolated from soil, which yielded 30 % **IV**.



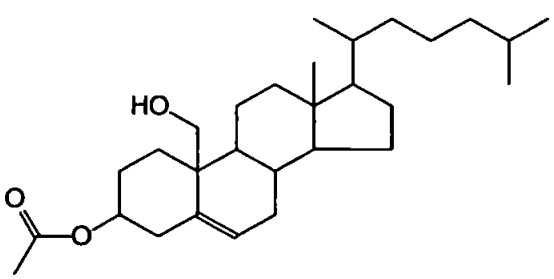
LXXIII



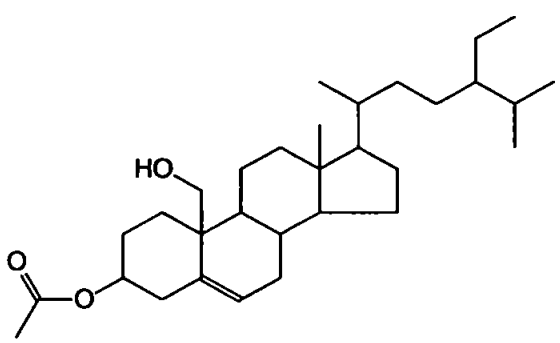
LXXIV

The production of **IV** was also demonstrated from androst-4-en-3,17-dione (**XXXXIX**). The aromatisation of the A-ring occurred *via* the insertion of a hydroxyl group on C19 methyl (**LXXIII**). The presence of the 19-hydroxyl group inhibited further oxidation of the sterol nucleus and resulted in cleavage of C19 and subsequent A-ring aromatisation (Bhattacharyya *et al.*, 1984).

The micro-organisms, *Moraxella*, isolated from soil also produced estrone (**IV**) from 19-hydroxy-androsten-3,17-dione (**LXXIV**) (Bhattacharyya *et al.*, 1984). **IV** was also produced from 19-hydroxy-3β-acetoxy cholest-5-ene (**LXXV**) and 19-hydroxy-3β-acetoxy sitost-5-ene (**LXXVI**) (Bhattacharyya *et al.*, 1984; Shankar *et al.*, 1993). The biotransformation of **LXXV** and **LXXVI** to **IV** has been shown to be mediated *via* 19-hydroxy-androst-4-en-3,17-dione (**LI**); no C22 phenolic acid intermediates were observed (Madyastha and Shankar, 1994).



LXXV

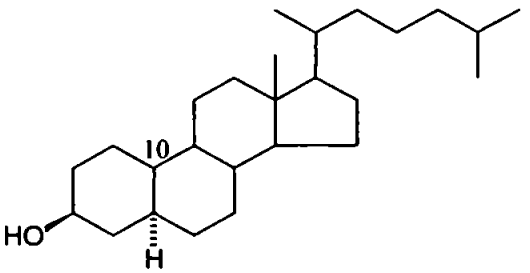


LXXVI

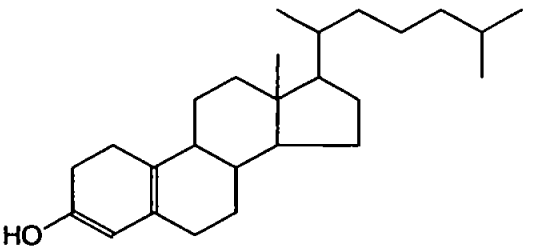
LXXII

In the Mediterranean sponge, *Axinella polypoides*, was used by Rabinowitz and Djerassi (1992) to examine products of ^{14}C cholesterol *in vivo*. The findings were similar to results obtained for bacterially-mediated breakdown of cholesterol (**III**) with the initial production of **LVII** and subsequent cleavage of the C19 methyl *via*

hydroxylation and oxidation to a carboxyl group. The end-product, 19-nor-5α-cholestan-3β-ol (**LXXIX**) was speculated to have been produced *via* a 19-nor-cholest-3,5(10)-dien-3-ol (**LXXX**) intermediate.

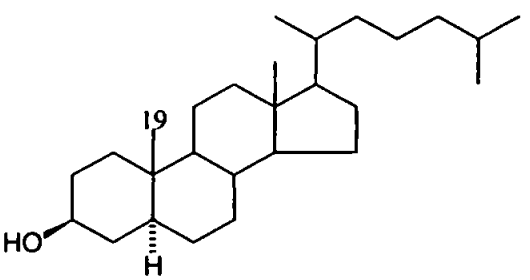


LXXIX

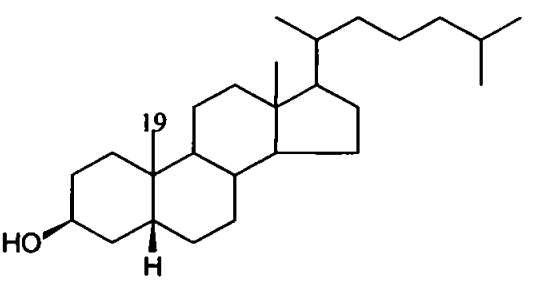


LXXX

Studies of **III** metabolism in the environment are even more sparse. Gaskell and Eglinton (1975) injected 4-^{14}C cholesterol into a freshwater sediment and also into anaerobic sewage sludge in order to investigate the production of stanols present in many sediments. The sediments were incubated *in situ* while the sewage sludge was incubated under ambient conditions in the laboratory. In sediments a small proportion of the cholesterol (**III**) was reduced to cholestanol (5α(H)-cholestan-3β-ol) (**LXXVII**) and coprostanol (5β(H)-cholestan-3β-ol) (**LXXII**) in the ratio 4:1 ratio. The **III** metabolism ratio in sewage sludge was 1:2, other degradation products of **III** were saturated and unsaturated 3-keto steroids (cholest-4-en-3-one (**LVII**)).



LXXVII



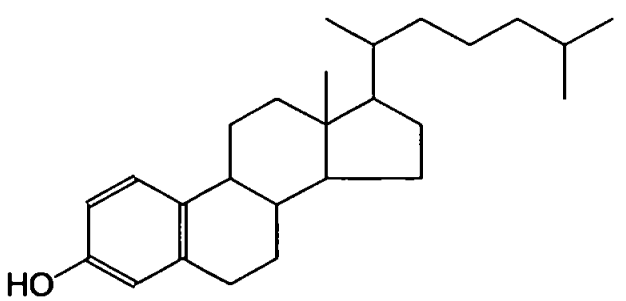
LXXII

Cholesterol (**III**) is usually the second most abundant sterol in STW effluents with concentrations ranging between 160-1890 μg L^{-1} and 27-360 μg L^{-1} in STW influent and effluent samples respectively (Quéméneur and Marty, 1994). Coprostanol (**LXXII**), produced in the intestines of higher mammals, is usually the most abundant sterol found in STW effluent and as such has been used to indicate sewage pollution in the environment (McCalley *et al.*, 1981). However, **LXXII** is not a reliable sewage pollution indicator as ^{14}C-cholesterol (**III**) incubated in freshwater sediments has been shown to reduce to **LXXII** (Gaskell and Eglinton, 1975). Elhmmali *et al.* (1997) identified that bile acids, synthesised in the liver from **III**, were present in sewage influent samples. The bile acids were surprisingly resistant to metabolism during sewage treatment, compared with **LXXII**, with concentrations in effluent samples relatively unaltered. Furthermore, the authors identified numerous bile acids in estuarine sediments adjacent to STW outfalls, thus suggesting that bile acids are a more reliable indicator of sewage pollution than **LXXII**. Further investigations have shown that bile acids were more resistant to metabolism in the environment when compared with **LXXII** (Elhmmali *et al.*, 2000).

1.6 Aims of the present study

From as early as the 1970s the analysis of STW effluents have identified that the natural and synthetic estrogens were present at alarmingly high concentrations. More recent studies have identified that STW effluents were indeed estrogenic and were primarily due to the presence of the natural estrogens (estrone (**IV**), 17β-estradiol (**I**) and 16α,17β-estriol (**V**)) and the synthetic estrogen, 17α-ethynylestradiol (**XIII**). The

source of the estrogens found in the effluent originated in the influent entering the STW. To date there has be no investigation to determine whether estrogen analogues can actually be produced as a result of sewage treatment (*i.e.* not all originating from the influent). Therefore, the aim of the present study was to investigate the hypothesis that estrogenic A-ring steroids in sewage treatment works effluents might originate not only from influent but also from *in situ* sources. Specifically it was hypothesised that abundant influent sterols, such as cholesterol (**III**) might be metabolised by microbial and higher organisms, to produce A-ring aromatic steroids estrone (**IV**) and 17β-estradiol (**I**) *via* intermediate analogues such as 19-norcholest-1,3,5(10)-trien-3-ol (**LXXXI**) (Figure 1.4).



LXXXI

From the foregoing review of steroid metabolism, this would appear to be feasible in organisms possessing the P_{450} cytochrome which is known to lead to A-ring aromatisation *via bis*-hydroxylation of the C-19 methyl group. The importance of such findings would be considerable given the potent estrogenicity of estrone (**IV**) in all bioassays and the huge influent burden of cholesterol into STWs. A sample of NCT (**LXXXI**) was thus synthesised herein to facilitate investigation of this pathway and specifically to obtain chromatographic and mass spectral data of the trimethylsilyl ether which are reported herein for the first time. The synthetic **LXXXI** also enables direct confirmation, if **LXXXI** was determined in environmental samples, by GC co-injection techniques. The estrogenic activity of the synthetic **LXXXI** to a commonly used yeast

bioassay was also determined. The compound was indeed weakly estrogenic. A careful study was then made of the products of incubation of radiolabelled (^{14}C)-cholesterol in simulated STWs under both aerobic and anaerobic conditions. Products of metabolism were then isolated and examined by radio TLC, radio HPLC and radio GC. The hypothesised pathway *via* **LXXXI** was not found to be important under the conditions tested but various new and known steroidal metabolites of cholesterol were detected, thus furthering the knowledge of steroid metabolism in STW.

Complementary to these studies, an improved analytical scheme for the routine monitoring of A-ring and other steroids in STW effluent was devised. The method involved SPE extraction of STW effluents, HPLC fractionation of compounds retained and subsequent GC-MS determination of fractions containing analytes of interest. In order to fully assess the potential environmental impact of STW effluents solids associated with the effluent were also extracted and analysed by HPLC and GC-MS. The method allowed numerous steroids, including estrone (**IV**), 17β-estradiol (**I**) and 16α,17β-estriol (**V**) to be monitored in major North London STW effluents where estrogenic (Vg production) effects had previously been observed in caged trout (Routledge *et al.*, 1998a). 16α,17β-estriol (**V**) which is highly estrogenic in all bioassays, has rarely, if ever, been determined in STW effluents previously. The concentrations of 17β-estradiol (**I**), estrone (**IV**) and 16α,17β-estriol (**V**) found dissolved in the effluent were in the range 0-6.4 ng L^{-1}. **IV** was the most abundant estrogen (>1 ng L^{-1}) whereas **I** and **V** were *ca* 1 ng L^{-1}. The concentrations of **IV** are in good agreement with previous concentrations reported. However the concentration of **I** was lower than previously reported from these STW effluents. Previous authors did

not report **V** in effluent samples analysed and **I**, **IV** and **V** were not determined in solid samples previously. In the present study NCT (**LXXXI**) was detected in only two solid samples taken from the same STW, indicating that it is a possible but uncommon product of STW metabolism.

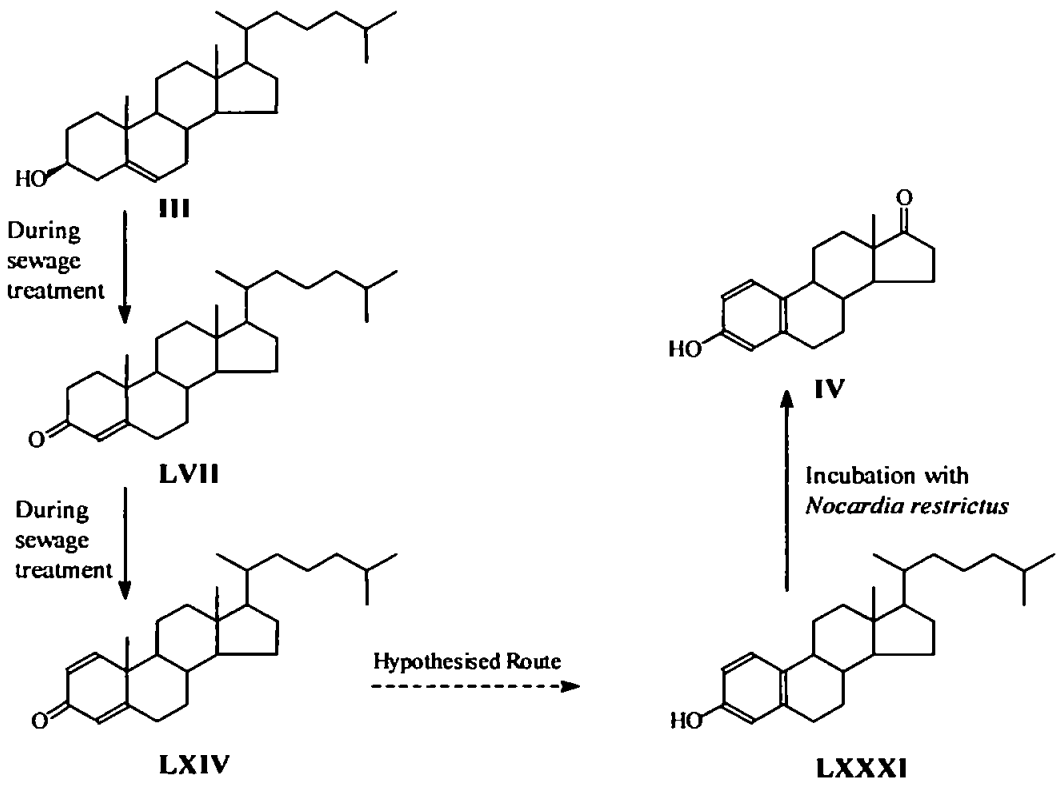


Figure 1.4 Hypothetical route for the formation of NCT (**LXXXI**) and estrone (**IV**) from cholesterol (**III**) during sewage treatment (Afonso *et al.*, 1966; Gaskell and Eglinton, 1975).

In summary, the specific aims of the present study were to:

- Synthesise NCT of high purity so it can be used as a reference compound for determining estrogenicity and LC and GC chromatographic properties.
- Examine the metabolism of ^{14}C cholesterol during aerobic and anaerobic sewage treatment for evidence of A-ring aromatisation and hence NCT production. Report metabolites identified.

- Optimise methodology for the detection of NCT in environmental samples.
- Monitor STW effluents, report environmental concentrations of NCT, natural female estrogens and other major steroids.

Chapter 2

Experimental Procedures

2.1 General laboratory procedures

Solvents used were all either HPLC or glass distilled grade (Rathburn Chemicals Ltd., Walkerburn, U.K.). Authentic reference steroids and reagents were purchased from Sigma-Aldrich Co. and Fluka and were the highest purity available. Glassware used throughout was soaked in 5 % Decon^{TM} solution for at least 24 h, rinsed (x 3) with Milli-Q water, dried in an oven at 110 °C and covered with hexane rinsed foil prior to use. Cotton wool, anhydrous sodium sulphate and sand were Soxhlet extracted with DCM for 24 h and stored in DCM rinsed glass jars covered with hexane rinsed aluminium foil. Silica gel and alumina used for column chromatography were Soxhlet extracted with DCM for 24 h and stored in an oven at 110 °C prior to use. Glass Pasteur pipettes were rinsed with DCM (x 3) before being used.

2.2 Instrumental details

2.2.1 Gas chromatography - mass spectrometry (GC-MS)

The GC-MS instrument used throughout the study was a Finnigan MAT GCQ. Details of the GC are shown below:-

Instrument	Finnigan MAT GC
Column	HP5-MS, 30m x 0.25mm i.d., 0.25 μm film
Injector	Split/splitless set to 250 °C
Carrier Gas	Helium at a constant velocity of 40 cm sec^{-1}
Oven Temperature Gradient	40 – 300 °C @ 10 °C min^{-1} with a 10 minute hold at 300 °C.

The MS conditions are shown below:-

Instrument	Finnigan Ion Trap Mass Spectrometer
Ionisation Energy	70 eV
Multiplier Voltage	1600 - 2200 V
Ion Source Temperature	180 °C
Transfer Line Temperature	295 °C
Scan Rate	2 scan sec^{-1}
Mass Spectrometer mode	Full scan, 40-600 Daltons

Sample injection was by autosampler. The conditions used are shown below:-

Instrument	CTC-200S autosampler
Sample volume	1 µL
Pre-solvent cleanings	3
Sample cleanings	1
Sample pullups	5
Solvent cleanings	5

2.2.2 Gas chromatograph-flame ionisation detector-radioactive detector (GC-FID-RD)

The GC-FID-RD was used in the monitoring of radioactive metabolites from incubation studies of ^{14}C cholesterol during sewage treatment. The details of the GC-FID are shown below:-

Instrument	Varian Star 3400 CX GC
Detector	Flame Ionisation Detector
Injector	On-column
Column	J&W DB5, 30 m x 0.35mm *i.d.*, 1 µm film
Carrier Gas	Helium
Oven Temperature Gradient	55 °C for 1 min., 55-100 °C @ 30 °C min^{-1}, 100 -300 °C @ 10 °C min^{-1} with a 10 min. hold

Eluting compounds were detected by FID and RD in a split ratio of 1:9. Details of the RD are given:-

Instrument	LabLogic GC-RAM radioactivity detector
Scintillation Gas	10 % Methane in Argon

2.2.3 High performance liquid chromatography (HPLC)

HPLC was used to fractionate complex mixtures of synthesised steroids and pre-concentrated STW effluents prior to GC analysis.

2.2.3.1 Normal phase HPLC conditions used for the purification of NCT

Pump	Merck-Hitachi 6200A intelligent pump
Detector	Merck-Hitachi L4200 UV/Vis spectrophotometer at 254 nm
Column	25cm x 4.6 mm i.d. silica, 10 μm bead size
Injection	Rheodyne 6 port injector with 100 μL loop
Mobile Phase	DCM at a flow rate of 1ml min^{-1}
Integration	Merck-Hitachi D2500 Chromato-Integrator

2.2.3.2 Reverse phase HPLC conditions used for the fractionation of concentrated sewage effluents

Pump	Merck-Hitachi L-6200A intelligent and L-6000 LC pumps
Detector	Merck-Hitachi L4200 UV/VIS spectrophotometer set to 210 nm
Column	25cm x 4.6 mm i.d. Hypersil, 10 μm bead size, ODS
Injection	Rheodyne 6 port injector with 250 or 2000 μL loop
Mobile Phase	Water and methanol gradient at a flow rate of 1ml min^{-1}
Integration	Merck-Hitachi D-2500 Chromato-Integrator

2.2.3.3 HPLC coupled with radioactivity detector

Pump	Dionex GP 40 Gradient Pump
Detector	Berthold LB 506 C-1 radioactivity monitor
Column	25cm x 4.6 mm i.d. Hypersil, 10 μm bead size, ODS
Injection	Rheodyne 6 port injector with 2000 μL loop
Integration	PC running Laura data capture, integration software

2.2.4 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H and ^{13}C NMR was performed on a Jeol EX 270 multinuclear 270 MHz spectrometer with $CDCl_3$. Chemical shift (δ) were referenced to tetramethyl silane (TMS).

2.2.5 Liquid scintillation counting (LSC)

Sub-samples of radioactivity extracted samples (up to 1 mL) were analysed by a Wallac 1400 Liquid Scintillation Counter using the EasyCount option for ^{3}H and ^{14}C isotopes. The minimum counts cut off was set to 0.00 cps and the counting time per sample set to 300 seconds. The scintillation cocktail used was ICN EcoLume™ which can take an aqueous loading of up to 40% (v/v).

2.2.6 External standard procedure

Prior to GC-MS analysis, HPLC fractions were spiked with a known amount of 1,4-^{2}H-17β-estradiol (98 atom % ^{2}H, Sigma-Aldrich Co.), in methanol, for determining the concentration of compounds of interest using calculated response factors.

2.2.7 Sample derivatisation with BSTFA

The solvent-free samples or authentic reference compounds (free alcohols) were converted to their trimethylsilyl ethers derivatives with BSTFA containing 1% TMCS. To each sample 20 µL (increasing to 50 µL depending on sample size) BSTFA:TMCS (99:1) in pyridine (1:2) was added and samples sealed. The samples were heated at 60 °C for 30 minutes, allowed to cool, and made up to 100 µL or 1000 µL with DCM.

2.2.8 GC-MS response factors

The response factor (RF, the ratio between the area of an external standard (ES) and the area of an authentic reference compound (ARC), both of known concentrations) was calculated by the following equation:

$$RF(ARC) = \frac{Area(ES) \times Amount(ARC)}{Area(ARC) \times Amount(ES)}$$

The response factors calculated were used to determine concentrations in environmental samples by re-arranging the equation (Desbrow *et al.*, 1998):

$$Amount(unknown) = \cdot \frac{Amount(ES) \times Area(unknown) \times RF(unknown)}{Area(ES)}$$

2.2.9 Kovats Retention indices

The GC retention indices (RI) were determined for all authentic reference compounds as the TMS ether derivatives. A series of approximately equimolar *n*-alkanes (C_{18}-C_{33}) was added to a mixture of authentic reference analytes and analysed by GC-MS (in triplicate). The RI were determined using the following equation:

$$RI = 100n + \left[\frac{logRT(ARC) - logRT(Cn)}{logRT(Cn+1) - log\ RT(Cn)} \times 100\right]$$

where RT = retention time in seconds

ARC = authentic reference compound

C_n = *n*-alkane with n carbon atoms eluting prior to ARC

C_{n+1} = *n*-alkane with n+1 carbon atoms eluting directly after ARC

The analytes examined all eluted in the 300 °C isothermal range on a GC temperature program and thus the above equation uses log values.

2.3 Synthesis of NCT

Cholest-1,4-dien-3-one (2 g) was synthesised from cholest-4-en-3-one by the method of Burn *et al.* (1960) and was kindly donated by Dr T. Peakman, University of Bristol. The identity and purity were determined by ^{1}H NMR prior to use (Chapter 3, Figure 3.6 page 73).

NCT was synthesised following the method of Afonso *et al.* (1966). Biphenyl (1.6 g) and clean lithium ribbon (72 mg) were added to purified (refluxed for 1 hour) THF (5 mL) in a round bottom flask, refluxed under nitrogen for 1 hour and allowed to cool. Cholest-1,4-dien-3-one (1 g), dissolved in purified THF (2.5 mL), was added to the mixture. The reactants were stirred for 30 minutes and the reaction quenched by the careful addition of Milli-Q water to remove any unreacted lithium. Ice was used to cool the reaction. The products of the reaction were extracted into ether (3 x 10 mL), rotary evaporated to dryness and stored at 4 °C in the dark prior to purification.

The initial purification stage was the removal of excess biphenyl by steam distillation. The biphenyl vapour was condensed and collected in a conical flask. The procedure was run continually until no further biphenyl condensed. The non-volatile solids remaining were re-extracted from the water into diethyl ether (x3). Both water and diethyl ether fractions were retained.

The reaction mixture (from diethyl ether fraction), dissolved in hexane, was applied to the top of a silica chromatography column (40 g) and compounds eluted by increasing the polarity of the eluting solvent:

E_1 (non phenolic compounds)	2 column volumes 1-5% (v/v) diethyl ether in hexane.
E_2 (phenolic compounds)	3 column volumes 10% (v/v) diethyl ether in hexane.
E_3 (remaining compounds)	3 volumes lengths of diethyl ether.

Fractions of the resulting eluent were collected and evaporated to dryness under a constant stream of nitrogen.

2.3.1 GC-FID determination of fractions

Fractions collected from the silica column were dissolved in 1 mL DCM. 1uL of each fraction was taken and further diluted to 1 mL with DCM. The raw fractions were initially analysed by GC-FID and the retention times compared with an authentic NCT reference compound (donated by Dr. Suiginome, University of Japan, Suiginome *et al.*, 1990).

Fractions containing >50% NCT were further purified by recrystallisation, HPLC and TLC fractionation. Fractions containing <50 % NCT were re-chromatographed over silica.

Impure NCT was recrystalised from pentane. The products obtained from the pentane recrystallisation were dissolved in DCM and separated by isocratic normal phase

HPLC (Section 2.2.3.1 for details). Product (100 μL) was injected and a fraction taken ± 2 minutes from the retention time obtained for an authentic NCT reference compound. The remaining unwanted products eluted were retained in a round bottom flask and the DCM removed by rotary evaporation. The solid mixture obtained was stored at 4 °C. The NCT fraction was blown down under a stream of nitrogen to ~50 μL and stored at 4 °C . The use of HPLC to separate NCT from by-products was time consuming and preparative TLC was introduced.

2.3.2 TLC cleanup

The glass TLC plates used, 25 cm x 25 cm, were degreased with acetone and allowed to dry. Silica gel (35 g, Merck, bead size 15 μm, containing a UV labelling compound for visualising at 254 nm) was made into a slurry by the addition of ~70 mL distilled water and shaken. The TLC plates produced were 0.5 mm in thickness and were stored in an oven at 110 °C for at least 1 hour before being used. The eluent used was DCM and prior to analysis the plates were pre-eluted (x 2) in order to remove any residual organic material. Plates were spotted with the 'NCT' HPLC fraction obtained using a glass capillary, along the length of the plate and eluted with DCM. Authentic NCT was also run for comparison. The R_f value was obtained by visualising the plate under UV light (254 and 364 nm). The region corresponding to NCT was retained, re-extracted into DCM, and retained. GC-MS analysis showed that the NCT required further purification due to the presence of keto steroids. To remove NCT from the co-eluting compounds NCT (as the TMS ether) was re-chromatographed over silica with DCM as the solvent phase. The region where NCT eluted was retained and analysed by GC-MS. The solid obtained was dissolved in ^{2}H tetrachloromethane for ^{1}H and ^{13}C NMR

determination. The final purity of the most purified aliquots was determined to be ~ 97 % (by GC-MS). However some larger batches were less pure (*ca* 85 %).

2.3.3 Theoretical octanol/water partition coefficient

The structure of NCT and two estrogenic steroids, 17β-estradiol and estrone, were used for the mathematical determination of the octanol/water partition coefficient (log K_{ow}) by Prof. J. Deardon, Liverpool John Moores University. Two methods were used, the Syracuse Research Corporation (SRC) and ClogP Medchem algorithms. The log K_{ow} values were determined using a fragmentation database.

2.3.4 Estrogenic potential using the yeast assay (YES)

The estrogenicity of NCT (10 mg) was determined using a genetically modified yeast (Glaxo Wellcome plc) by the method of Routledge and Sumpter (1996). The modified yeast contained the DNA sequence of the human estrogen receptor that has been incorporated into the main genome. The response to estrogenic compounds is the production of β-galactosidase which reacts with the chromogenic substate chlorophenol red-β-galactpyranoside (CPRG) producing a characteristic red colouration (Environment Agency Report, 1997; Routledge *et al.*, 1998a).

2.4 Sewage treatment works effluent monitoring

2.4.1 Sampling sites

Effluent samples were taken from two Thames Water plc STWs, Harpenden and Deephams, (see Figure 5.1, page 150) both of which discharge into the river Lea, Hertfordshire. The type of treatment undertaken at these works is shown below, along with the daily flow and size of catchment areas (Table 2.1). Sampling locations were

the sites of previous research which has demonstrated that the STW effluents are estrogenic as measured by vitellogenin (Vg) production in caged rainbow trout (Desbrow *et al.*, 1998)

Site	Domestic Population	Daily Flow (m^3)	Treatment Applied
Harpenden	31 200	8 250	Secondary, percolating and sand filters
Deephams	796 000	160 000	Diffused air activated sludge

Table 2.1 Details of STWs from which effluents were obtained and examined.

2.4.2 On site sampling of effluents

Sampling of sewage effluents followed the procedure of the Environment Agency Report (1997) and Desbrow *et al.* (1998). Prior to sampling, all equipment was disinfected in a weak 5 % (v/v) solution of bleach for 24 h and rinsed with Milli Q water (x 3). Methanol was used as a final degreasing agent.

Effluent samples (10 L) were collected from STW effluents using a stainless-steel bucket and poured into 10 L glass carboys, containing 50 mL of methanol as organic modifier, *via* a stainless-steel funnel. Samples were taken between February and August 1998 at approximately 1400 h

Generally extraction of the effluent samples onto SPE cartridges was started within 5 h of samples being taken. On the rare occasions where this was not possible the samples were stored in a constant temperature room held at 6 °C for a maximum of 10 h.

2.4.3 On site sampling of solids

Solids in the effluents were sampled at the same time as sampling of liquid samples. The volume of water filtered to obtain a workable solid sample depended on solids loadings of individual STW. In general, 20-50 L of effluent was adequate. The sampling procedure followed that outlined in Section 2.4.2. The effluent, however, was passed through a 10 μm pore size nylon mesh that retained the majority of solid material, and the liquid was discarded. The solids retained were removed from the mesh *via* a micro spatula and placed into a 6 mL glass vial containing 2 mL methanol and shaken. Samples were stored at - 20 °C prior to extraction.

2.4.4 Extraction of effluent by SPE

The dissolved organics in the effluent were extracted onto 5 g ODS (endcapped), SPE cartridges (Jones Chromatography). Cartridges were pre-cleaned/conditioned by application of a slight vacuum and drawing through 10 mL methanol. This was followed by 2 x 10 mL Milli-Q water and care was taken not to allow the cartridge to dry out. An in-line filter of 2 x 20 μm cellulose acetate filters sandwiching a GFC filter and a plug of cotton wool was fitted ahead of the cartridge to remove any solid material (Figure 2.1).

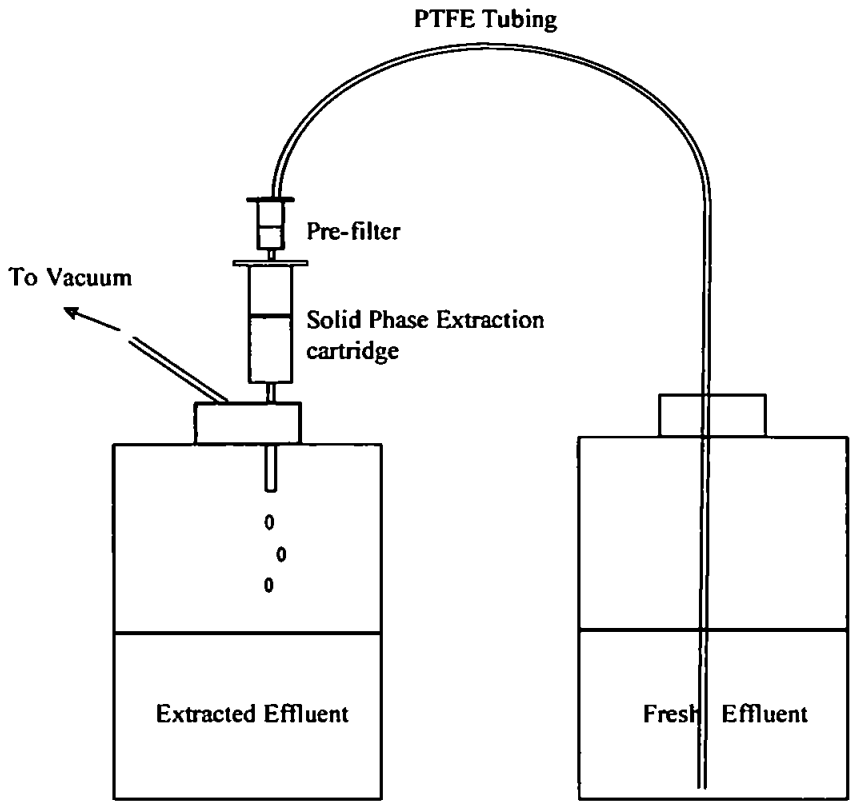


Figure 2.1 Apparatus used for SPE of effluent samples

The SPE cartridge was fitted into a 20 L glass carboy as a holding reservoir for extracted effluent, which was connected to a vacuum pump. The SPE cartridge was connected to the inline filter which in turn was fitted with a length of PTFE tubing that was submerged in the raw effluent. When the vacuum was applied the effluent was drawn through the pre-filter and cartridge. The flow rate was regulated by a needle valve at <10 mL min^{-1} since a greater flow reduced the efficiency of the cartridge. The extraction generally took ~20 h to complete. On completion, cartridges were dried by the vacuum pump drawing air through until water stopped flowing. The cartridge and inline filter were removed, wrapped in hexane-rinsed aluminium foil and stored at -20 °C prior to analysis.

2.4.5 Extraction of solids by ultrasonication, centrifugation and filtration

The extraction of solid samples was achieved using a modification of the U.S. EPA method outlined by Nguyen *et al.* (1995). The SPE in-line filter solids and directly-obtained solid samples were extracted in the same way. Samples were defrosted at room temperature. The samples were extruded into 6 mL glass vials containing 2 mL methanol. The samples were ultrasonicated for 15 minutes. After extraction the samples were centrifuged at 3500 rpm for 20 minutes, the supernatant decanted off, filtered through cotton wool and retained. The entire procedure was repeated twice using methanol followed by DCM. The three extracts were combined and blown down to dryness under a gentle stream of nitrogen and stored at -20 °C prior to analysis by GC-MS.

2.4.6 Elution of organic material from SPE cartridge

The fractionation procedure of the effluent samples was a modification of the Environment Agency Report (1997) and Desbrow *et al.* (1998). When required, the SPE cartridges were allowed to defrost at room temperature for 2 h. The organic material was eluted from the cartridge using a SPE vacuum manifold with 2 x 15 mL hot (60 °C) methanol and collected in a methanol rinsed round bottom flask. The amount of methanol was reduced to *ca* 2 mL by rotary evaporation. The sample was decanted into a 6 mL glass vial. The round bottom flask was rinsed with methanol (x3) and combined with the sample. The sample was further concentrated to ~ 1 mL under a steady stream of nitrogen and stored at 4 °C prior to HPLC fractionation.

2.4.7 Fractionation of organic material by HPLC

The organic material eluted from the SPE cartridge was fractionated by a reverse phase HPLC gradient system as described in detail in Section 2.2.3.2).

The mobile phase was a gradient mix of water and methanol in the following proportions (Table 2.2). These are a slight modification of the conditions described by Environment Agency Report (1997) and Desbrow *et al.* (1998). The flow rate was 1 mL min^{-1}.

Time (minutes)	Methanol (%)
0-3	10
3-30	10-100
30-45	100
45-50	100-10

Table 2.2 Gradient mix used for HPLC fractionation of effluent extracts.

Before the fractionation of the effluent extract a series of authentic reference compounds, 17β-estradiol, estrone, 16α,17β-estriol and NCT, were analysed in order to determine relative retention times. The injector was flushed with excess methanol after every reference compound to avoid contamination. Methanol procedural blanks were also obtained and fractions collected for GC-MS analysis to assure no cross contamination with reference compounds.

Extracted effluent (4 x 250 μL) was examined by HPLC and fractions collected at 1.5 minute intervals from 15 minutes to 45 minutes within a single run. The equivalent fractions from different runs were pooled and solvent removed under a constant stream of nitrogen. The samples were stored in the dark at 4 °C before analysis by GC-MS.

2.4.8 GC-MS determination of liquid and solid samples

Prior to GC-MS analysis the fractions were spiked with an external standard, 1,4-^{2}H-17β-estradiol, derivatised with BSTFA and 1 μL sample injection. Before a sequence of samples were analysed two full procedural DCM blanks were obtained.

2.5 Cholesterol metabolism studies

2.5.1 ^{14}C cholesterol metabolism with activated sewage sludge

Examination of the aerobic metabolites of 4-^{14}C and 26-^{14}C cholesterol (American radiolabelled Chemicals Inc) during laboratory scale wastewater treatment was studied using a semi-continuous activated sludge (SCAS) unit (Zeneca, PLC) (Boerhling *et al.*, 1997) or a simpler die away (DA) procedure (method from Dr. J. Snape, Brixham Environmental Laboratory, Zeneca).

2.5.2 The SCAS system

The SCAS unit consisted of a 1 L aeration chamber closed at its base by a glass sinter through which air was drawn (*via* a Drechsel bottle containing 2M NaOH to remove atmospheric CO_2) at 1.5 L min^{-1} (Figure 2.2). A small diaphragm vacuum pump connected to the top of the chamber created the negative pressure required to draw air up. Connected in-line between the aeration chamber and the pump were a series of two Drechsel bottles containing 2 M NaOH solution to trap $^{14}CO_2$. The main chamber had a side tap about 8 cm above the sinter through which liquid could be removed for analysis.

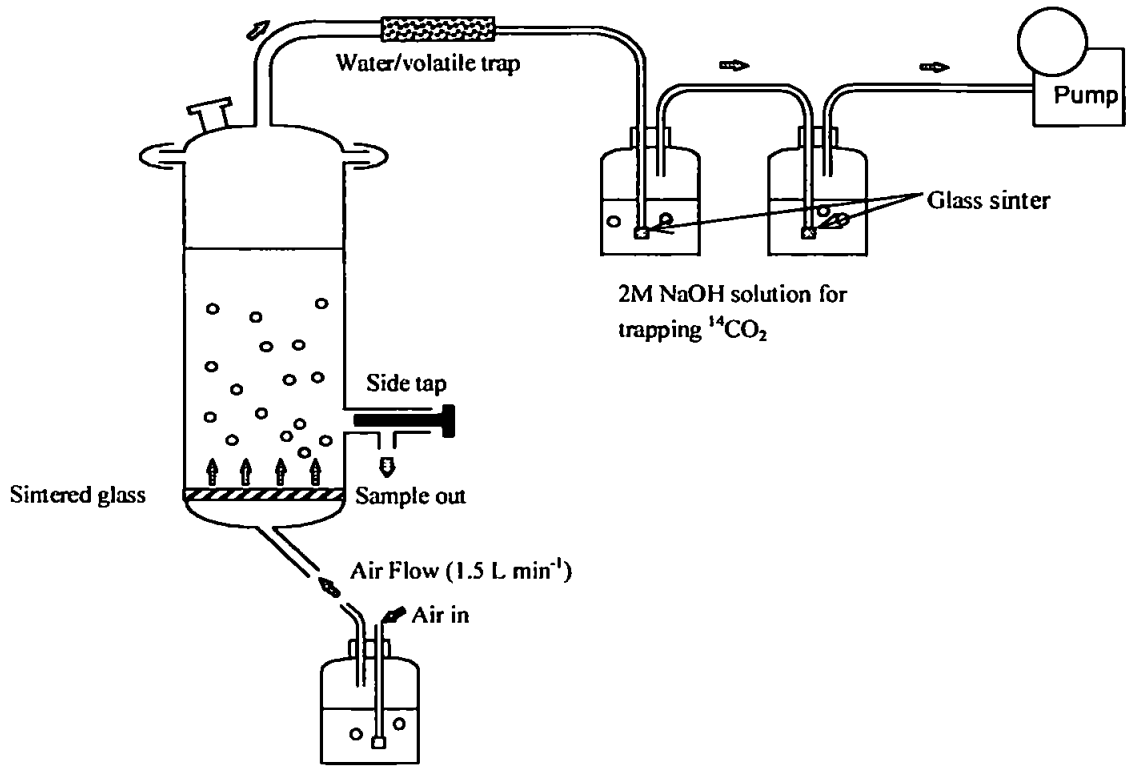


Figure 2.2 The operational SCAS unit.

The aeration chamber contained 600 mg activated sludge 1 L sewage influent (Newton Abbot STW) and 1 mL synthetic feed (a mixture of bactopeptone, lab lemco and salts (NaCl, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, K_2HPO_4 and urea)) and was aerated with CO_2 free air (prior to the addition of 4-^{14}C-cholesterol). Aeration was stopped after 23 h and 100 mL of liquid with solids immediately drawn off, of which 25 mL were filtered through oven prepared (110 °C) GFC filters, dried to constant weight and total solids determined gravimetrically. The remaining solids in the unit were allowed to settle over 1 hour after which 400 mL of liquid were removed. Total carbon was determine on 100 mL and the remaining 300 mL was discarded. To the unit 500 mL of fresh influent was added and the system returned to aeration. This procedure was repeated every 23 h in order to keep the micro-organisms in the SCAS working optimally.

For the incubation experiment 33 μL (49 KBq) of 4-^{14}C cholesterol in ethanol was added to the SCAS and the unit aerated. 100 mL of liquid and 60 mg of solids were taken every 24 h over a 4 day period. The liquid samples were frozen before extraction. The solid samples were centrifuged at 3500 rpm for 10 minutes, the supernatant decanted off, retained, 5 mL methanol added, shaken and stored at -20 °C. Liquid samples were extracted as described in Section 2.4.4. The final volume of the extract was 2 mL of which a 200 μL was taken for direct LSC determination of radioactivity. Solid samples were extracted as described in Section 2.4.5 except methanol (5 mL) was the solvent used throughout. Aliquot of each extract (1 mL) were taken for LSC. The remaining extract volume was reduced to *ca* 2 mL under a gentle stream of nitrogen.

2.5.3 The die away (DA) system

The DA experiment was similar to the SCAS system (Figure 2.3). The raw material was fresh influent (1 L) aerated in a 1 L measuring cylinder. Synthetic feed was not added in this case and hence the micro-organism population was not maintained and therefore could only be used optimally for 48 h. Two 100 mL 2M NaOH traps were used inline to adsorb $^{14}CO_2$. Two systems were run in parallel, one for 24 h, the other for 48 h. An abiotic control was also run.

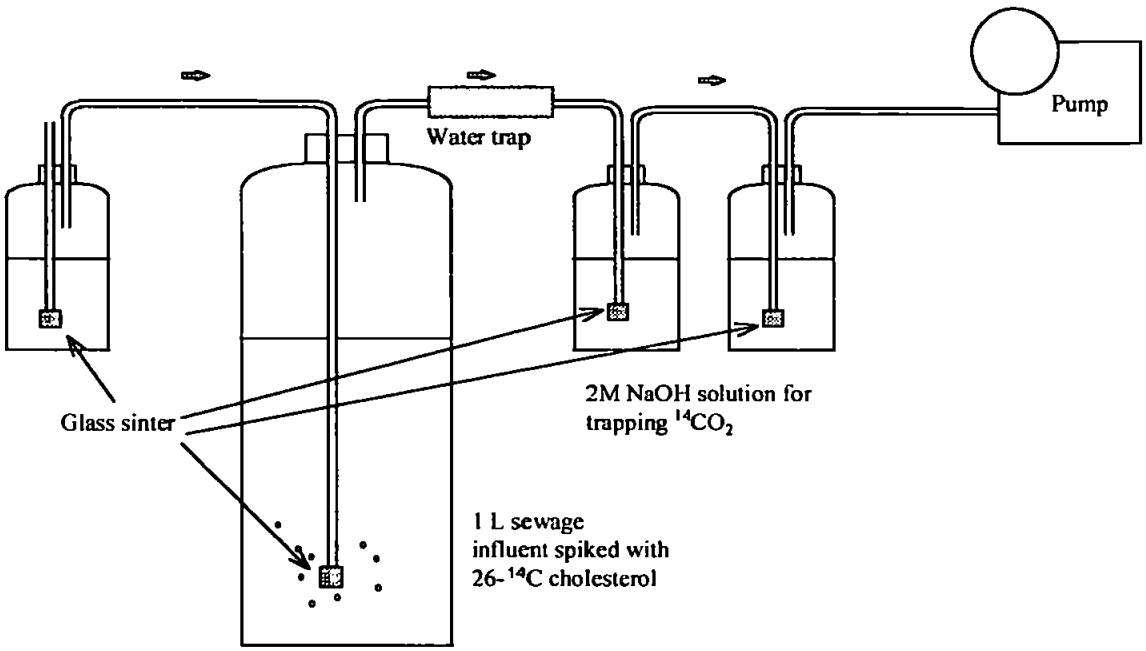


Figure 2.3 The operational DA system.

The systems were connected up and initially aerated for 1 h before being spiked with 26-^{14}C-cholesterol. To both systems 200 μL (740 KBq) ^{14}C cholesterol was added and the units aerated for the desired length of time. Aeration was stopped after 24 h intervals and the liquid divided into 4 x 250 mL of which 250 mL was filtered through GFC, dried and used to determine the amount of solids. The remaining sub-samples were centrifuged at 3500 rpm for 30 minutes and the supernatant decanted off for SPE extraction. The solid samples were dispersed into 8 mL methanol and stored at -20°C prior to extraction.

For abiotic controls the system described previously was used. Prior to the addition of the 26-^{14}C cholesterol the micro-organisms in the liquor were inhibited by the addition of mercuric chloride (75 mg) and allowed to aerate for 1 h after which the radiolabelled cholesterol was added.

The extraction procedure (liquid and solid) described in sections 2.4.4 and 2.4.5 were used. The extracts were stored at -20 °C

2.5.4 $^{14}CO_2$ determination by LSC

The radioactivity of primary and secondary traps from the SCAS and DA units were counted by LSC to determine the rate at which cholesterol had been mineralised (detail of LSC described in Section 2.2.5). From each Drechsel bottle a 5 mL aliquot was taken and dispensed into a liquid scintillation vial containing 10 mL scintillation cocktail. The samples were left in the dark for 1 h to allow any chemiluminescence to subside.

2.5.5 Radio-HPLC fractionation of samples

The liquid and solid sample extracts were fractionated by radio-HPLC (rHPLC) as outlined in Section 2.2.3.3. The mobile phase was that used by Desbrow *et al.* (1998) (Table 2.3).

Time (minutes)	Methanol (%)
0-3	40
3-30	40-100
30-45	100
45-50	40-100

Table 2.3 The methanol/water gradient mix used for the r-HPLC fractionation of ^{14}C cholesterol incubation studies.

The efficiency of the detector was assessed by analysis of a series of ^{14}C cholesterol standards of known activity in methanol. Procedural blanks were run prior to samples.

Sample fractionations were collected wherever radioactive peaks were detected and these were then dried under a stream of nitrogen. The fractions were stored under argon at -20° C. Radio-TLC (rTLC) (silica, DCM) was employed as a further clean up step for selected fractions. Detection was by a Berthold automatic scanning TLC detector using methane as counting gas. The sensitivity was generally set to 100 - 1000 cps and the scanning rate was 300 mm h^{-1}. Areas where activity was detected were retained for radio-GC (rGC) analysis.

Fractions obtained during rHPLC and rTLC were analysed by rGC (details in Section 2.2.2) as the TMS ether derivatives. A series of ^{14}C cholesterol references (as TMS ether) were used to optimise the split ratio and to determine the lag between the FID and RD responses and the efficiency of the RD. Prior to analysis of fractions excess BSTFA:pyridine was removed from all samples under a stream of nitrogen and samples re-dissolved in as little DCM as possible. Blanks and reference samples (1 μL) were analysed first. *n*-Alkane series were also examined to determine retention indices prior to GC-MS analysis.

2.5.6 Anaerobic metabolism studies of cholesterol

To each of twenty seven 120 mL serum bottles anaerobic activated sludge (2 g) was added and made up to 100 mL with water. The serum bottles were sealed and allowed to become anoxic. To each bottle 4.9 KBq 4-^{14}C cholesterol was added and samples incubated at 35 °C. The bottles were shaken on a daily basis. Once a week 3 bottles were made abiotic by the addition of 5 mg mercuric chloride and removed from the incubator.

When required serum bottles were opened and the contents centrifuged at 3500 rpm for 30 minutes to separate the solids from the liquids. The liquid portion was decanted off and retained. Both liquid and solid samples were either extracted immediately or stored at -20°C. The liquid samples were extracted as described in Section 2.4.4. Prior to the extraction of solid samples they were initially frozen and freeze dried. The solid samples were extracted with DCM (x 4) as described in Section 2.4.5. The extracts were fractionated by rHPLC, followed by rGC and GC-RD as described in Section 2.5.5.

To increase unit activity and to remove unwanted compounds, a high proportion of solid extract samples were further fractionated by rTLC in order to pre-clean the extracts (described in Section 2.5.5). Radioactive components, especially in the R_f region of NCT, a putative intermediate in the conversion of cholesterol to estrone, were extracted and blown down under nitrogen. The fractions were re-chromatographed over silica using acetone:hexane (1:1) and again analysed by rTLC. The radioactive components were retained for rGC and GC-MS analysis.

Chapter 3

Synthesis of a putative intermediate in the bacterially-mediated conversion of cholesterol to estrone: 19-norcholest-1,3,5(10)-trien-3-ol (NCT)

Aim

A major goal of this study was to determine whether cholesterol could be converted to estrone during sewage treatment *via* the intermediate compound 19-norcholest-1,3,5(10)-trien-3-ol (NCT) (**LXXXI**). The aim of the work described in this chapter was therefore to synthesise **LXXXI** of the highest purity possible *via* a known route. The NCT produced would then be used to determine chromatographic and mass spectral properties. These have not been reported previously and will act as an aid to the determination of **LXXXI** in STW effluents *via* the use of co-injection techniques. Finally, **LXXXI** is believed to have an estrogenic potential (although not determined) and thus will be tested *via* an established method.

3.1 Introduction

Within the mammalian P_{450} cytochrome system the biosynthesis of estrogenically active 19-nor steroids, such as estrone (**IV**) and 17β-estradiol (**I**), occurs *via* cholesterol-derived androst-4-en-3,17-dione (**XXXXIX**) or testosterone (**XVI**) substrates (Devlin, 1997). The loss of the C19 methyl group from position C10 occurs *via* an enolisation of the keto group and *bis*-hydroxylation at C19 (Devlin, 1997). The subsequent removal of the methyl group creates aromatisation of the A-ring. The biochemical importance of this route has meant that the chemical synthesis of A-ring aromatic steroids such as estrone, has also been examined by a number of authors (Dryden *et al.*, 1964; Afonso *et al.*, 1966; Sengupta *et al.*, 1987; Suginome *et al.*, 1988;

Kocovský and Baines, 1994). Dryden *et al.* (1964) described a method by which a diendione such as androst-1,4-dien-3,17-dione-17-ethyleneketal (**LXXXXVII**) could successfully accept two electrons on the A-ring and in so doing lead to aromatisation of the A-ring with the expulsion of C19 to form estrone (**IV**) (Figure 3.1).

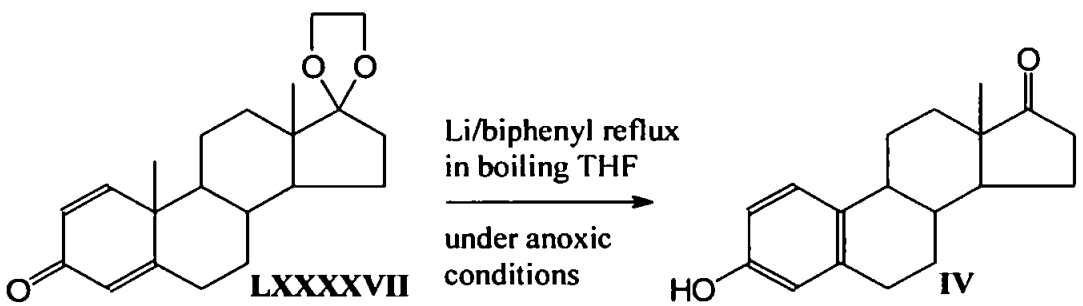


Figure 3.1 A-ring aromatisation of androst-1,4-dien-3,17-dione-17-ethyleneketal (**LXXXXVII**) to produce estrone (**IV**).

3.2 Reaction mechanism

Dryden *et al.* (1964) described the reaction mechanism for the synthesis of A-ring aromatic steroids. In order for the reaction to occur the reaction chamber had to be void of oxygen (*i.e.* under constant stream of high purity nitrogen) and all reagents moisture free. Initially the biphenyl, lithium wire and THF were added to the reaction chamber and then heated under reflux for 1 h. The lithium wire and biphenyl react to produce the corresponding lithium salt (Figure 3.2).

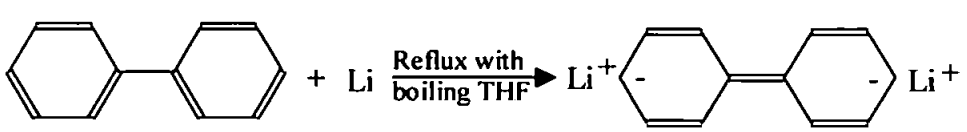


Figure 3.2 Production of the dianion electron donor from lithium wire and biphenyl in THF under moisture-free and oxygen-free conditions.

Sodium and potassium can also be used to form the dianion metal salt, but lithium is more stable with a higher binding strength (Holy, 1974). Dryden *et al.* (1964) replaced lithium with sodium and potassium in their experiments but discovered that the yield of estrone (**IV**) was lower. The electron donor system works in a similar way to the Birch reaction but without a proton donor present, such as an alcohol, producing a cyclohexadiene (March, 1985). The starting material, cholest-1,4-dien-3-one (**LXIV**) in THF, was added and stirred for 30 minutes. The steroidal dienone, **LXIV**, accepts an electron forming an anion. The negative charge on the keto group is unstable and thus the keto double bond is cleaved allowing the formation of the phenoxide A-ring and removal of the angular methyl. The C19 methyl forms the corresponding methyllithium (Dryden *et al.*, 1964). The overall reaction is shown in Figure 3.3.

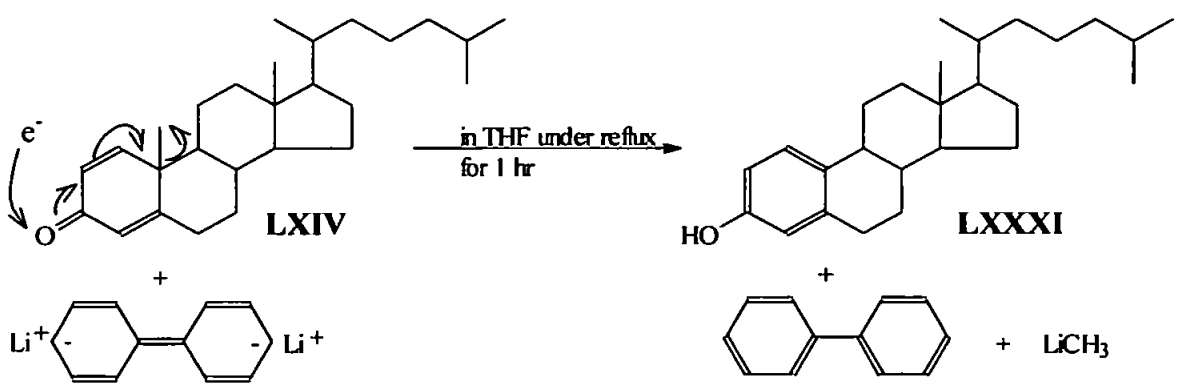


Figure 3.3 The overall reaction and mechanism between the dianion electron donor and the reactant, cholest1,4-dien-3-one (**LXIV**), to synthesis NCT (**LXXXI**).

The mechanism is thus quite unlike that known to occur in biota were the methyl is cleaved prior to aromatisation. Afonso *et al.* (1966) adapted the above method by aromatising cholest-1,4-dien-3-one (**LXIV**) producing NCT (**LXXXI**) 40 % yield.

Afonso and co-workers also showed that **LXXXI** could be microbially metabolised into estrone (**IV**) by the bacterium *Nocardia restrictus* (8 % conversion in 240 h). Subsequently modifications have been made to the original synthetic procedure of Afonso *et al.* (1966) and in order to increase the yields other methods have also been developed (Cambie *et al.*, 1969; Lack and Ridley, 1970; Kocovský and Baines, 1994). Recently Bannister *et al.* (1998) demonstrated that the dienone 17β-(*t*-butyldimethylsilyloxy)-12,12-ethylenedioxy-androsta-1,4-dien-3-one (**LXXXXV**) could under-go A-ring aromatisation, using the procedure of Dryden *et al.* (1964) to produce 17β-hydroxy-12,12-ethylenedioxyestra-1,3,5(10)-trien-3-ol (**LXXXXVI**) (Figure 3.4).

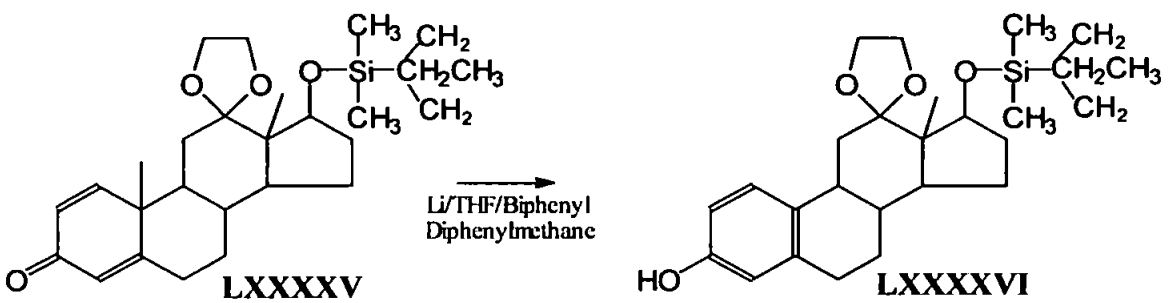


Figure 3.4 A-ring aromatisation of 17β-(*t*-butyldimethylsilyloxy)-12,12-ethylenedioxy-androsta-1,4-dien-3-one (**LXXXXVI**)

In the present study NCT (**LXXXI**) was synthesised by the method of Afonso *et al.* (1966) using cholest-1,4-dien-3-one (**LXIV**) as the starting material (Figure 3.5). **LXIV** was produced from cholest-4-en-3-one (**LVII**) using the method of Burn *et al.* (1960). The **LXXXI** was used to accurately determine previously unreported chromatographic (HPLC and GC) and mass spectral properties and for estrogenicity and environmental monitoring purposes the NCT (**LXXXI**) was required to be of highest purity possible.

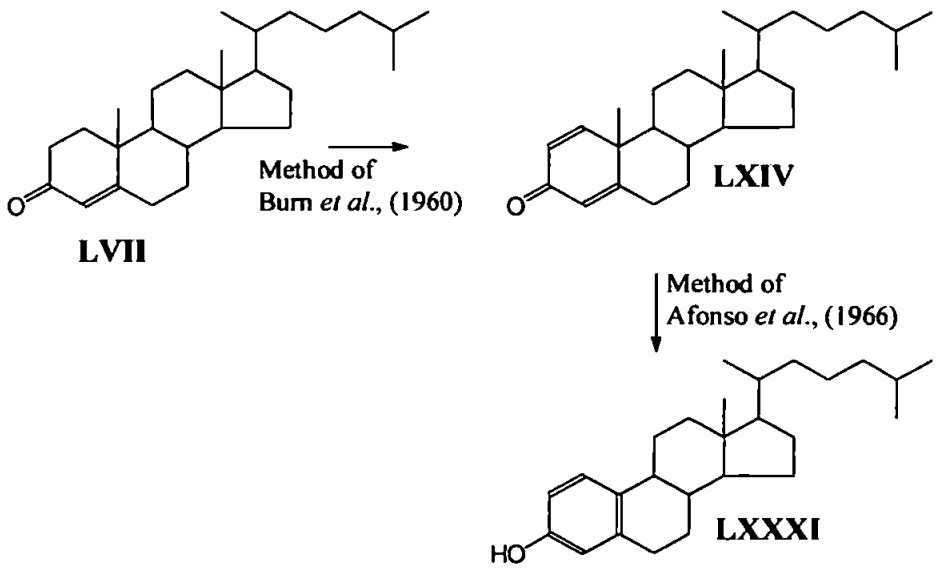


Figure 3.5 Overall reaction for the synthesis of NCT.

3.3 Results of synthesis

The starting material, cholest-1,4-dien-3-one (**LXIV**) was kindly synthesised and donated by Dr. T. Peakman, University of Bristol. The identity was checked by ^{1}H NMR (Figure 3.6) and was entirely consistent with the structure.

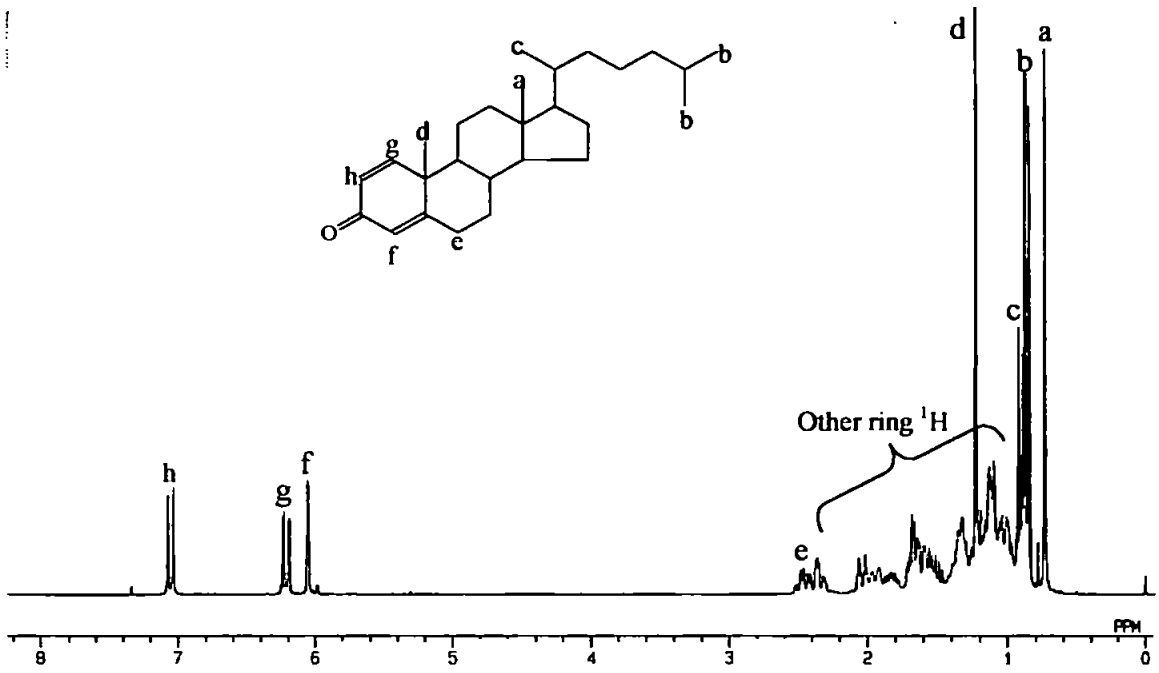


Figure 3.6 ^{1}H NMR spectrum of the starting material cholest-1,4-dien-3-one.

The initial green colouration of the biphenyl lithium in THF changed to an intense orange upon addition of the dienone to biphenyl, lithium in THF. The reaction was allowed to stand for 30 minutes prior to quenching by the careful addition of water to remove unreacted lithium. The products of the reaction were extracted into diethyl-ether (x 3). The aqueous and ether layers were both analysed by GC-MS. The aqueous yellow layer contained no analytes of interest while the diethyl-ether layer contained the NCT (**LXXXI**) and by-products of the reaction (Figure 3.7).

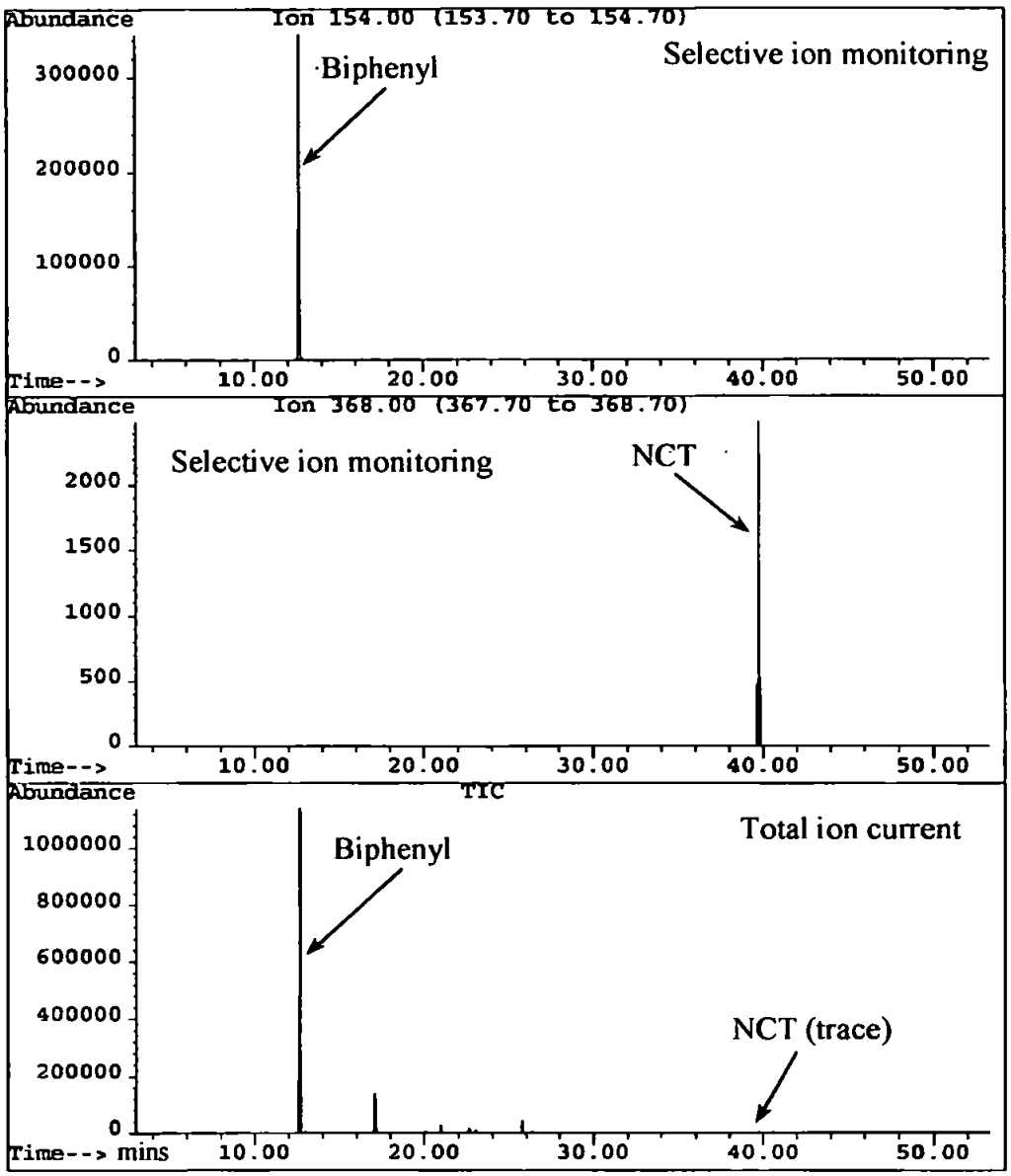


Figure 3.7 GC-MS data for orange diethyl-ether layer of crude reaction products of cholest-1,4-dien-3-one aromatisation showing the total ion current and selective ion monitoring of the molecular ions of NCT (*m/z* 368) and biphenyl (*m/z* 154).

The diethyl-ether was removed by rotary evaporation and the orange solid was steam distilled to remove free biphenyl. The remaining products were again extracted into diethyl-ether and chromatographed over 95 % activated silica gel. The products were

eluted with diethyl-ether in hexane (1 %, 3 % and 5 % diethyl ether in hexane) was used to elute non-phenolic compounds, 10 %, was used to elute phenolic compounds (Afonso *et al.*, 1966). The majority of NCT (**LXXXI**) eluted after 160 mL of 10 % diethyl-ether in hexane had been passed through the column (Figure 3.8).

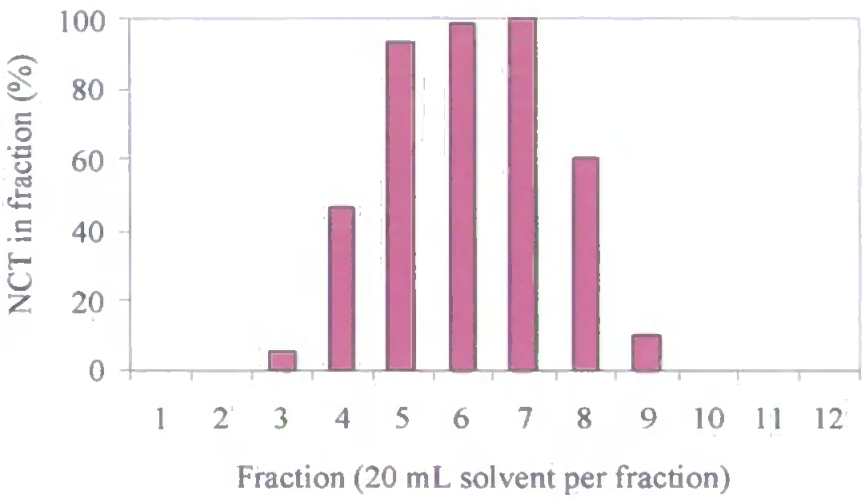


Figure 3.8 The elution of NCT with 10 % diethyl-ether in hexane from a silica gel column. Percentage **LXXXI** in each fraction was determined by GC-MS.

Finally the column was flushed with 50 % diethyl ether in hexane and 100 % diethyl ether in order to elute all other reaction by-products. **LXXXI** was only present in the 10 % diethyl-ether fractions. A small amount of cholest-4-en-3-one (**LVII**) was also present in the same fractions (Figure 3.9).

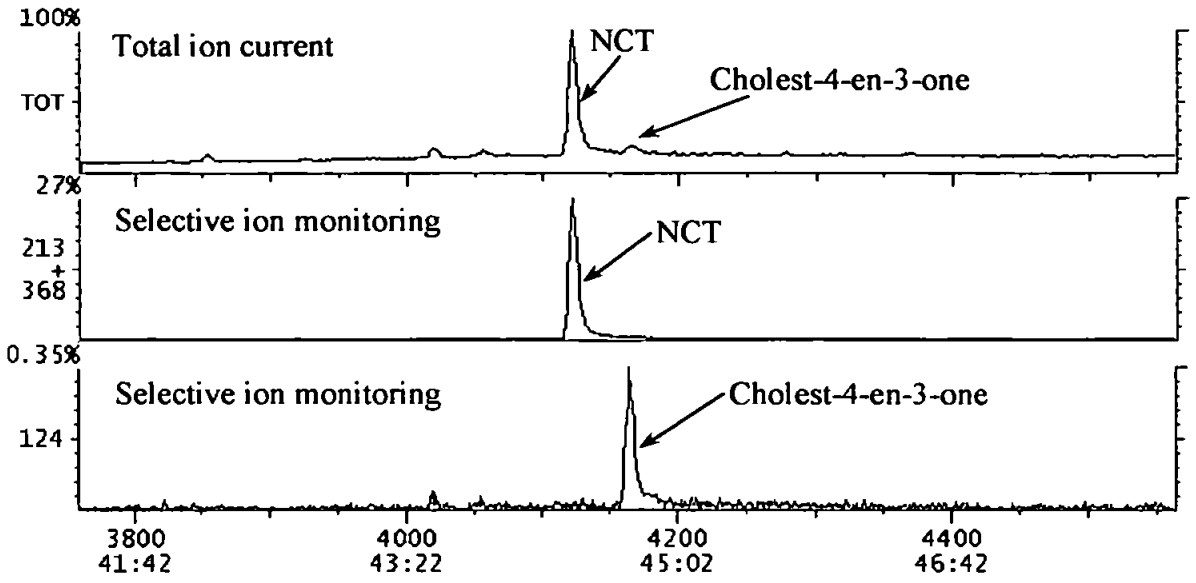


Figure 3.9 Diethyl-ether fraction (10 %) containing NCT (*m/z* 213+368) and cholest-4-en-3-one (*m/z* 124). The starting material, cholest-1,4-dien-3-one was not observed.

In the attempt to further separate NCT (**LXXXI**) from the keto steroid the products were re-crystallised in pentane (Afonso *et al.*, 1966) but little improvement in purity was obtained. Analytical TLC was investigated (100 % activated 0.5 mm thick silica gel on glass plates) using a range of mobile phases to purify the **LXXXI** (Table 3.1).

Mobile Phase	**R_f NCT**
5 % diethyl-ether in hexane	0
10 % diethyl-ether in hexane	~ 0.1
Methanol	0.9-1.0
DCM	0.37

Table 3.1 TLC retardation factor (R_f) of NCT on silica with four mobile phases.

Given the results shown in Table 3.1, DCM was adopted for preparative TLC. TLC-purified NCT was re-extracted into methanol and examined by GC-MS and ^{1}H ^{13}C NMR. These analyses revealed the presence of coprostan-3-one (Figure 3.10).

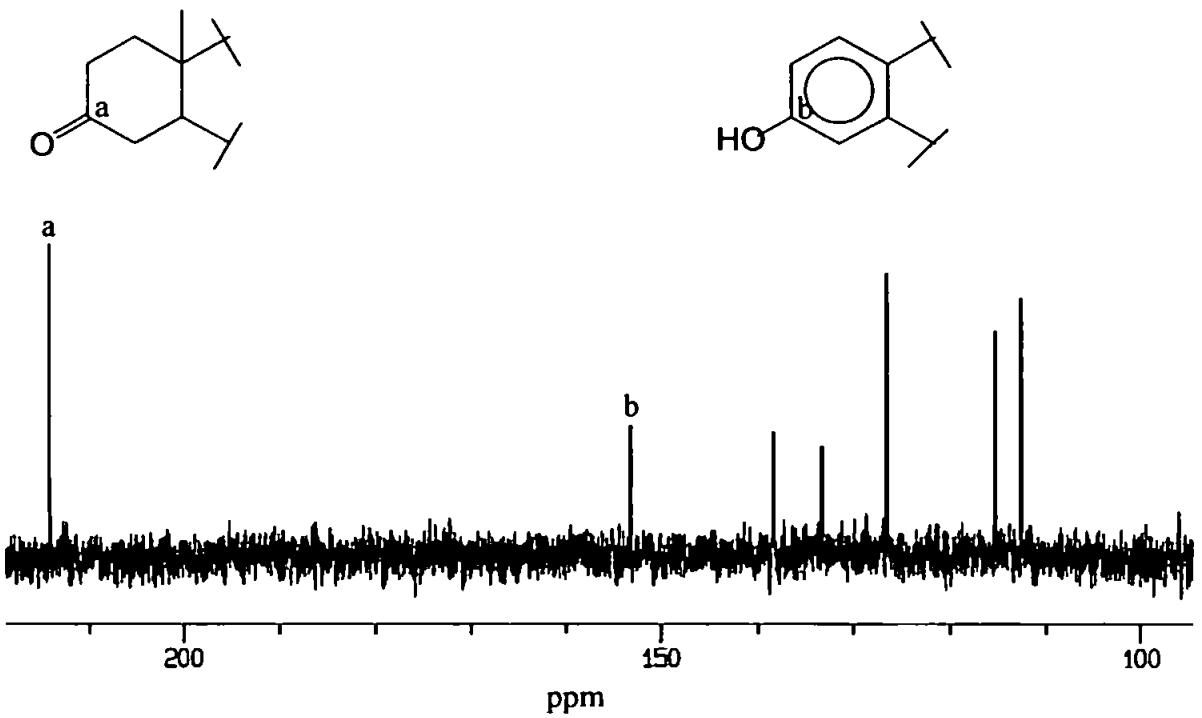


Figure 3.10 ^{13}C NMR spectrum of NCT showing the presence of a keto carbon and the six aromatic carbons of NCT.

NCT was further purified by TLC of the TMS ether on silica using DCM as the mobile phase (R_f NCT TMS = 0.82, coprostan-3-one ≈ 0.30). The ^{1}H NMR spectrum of the purified NCT is shown in Figure 3.11 and ^{13}C NMR detailed in Table 3.2. The carbonyl impurities were no longer present as reflected by the absence of the carbonyl carbon from the ^{13}C NMR spectrum (Table 3.2).

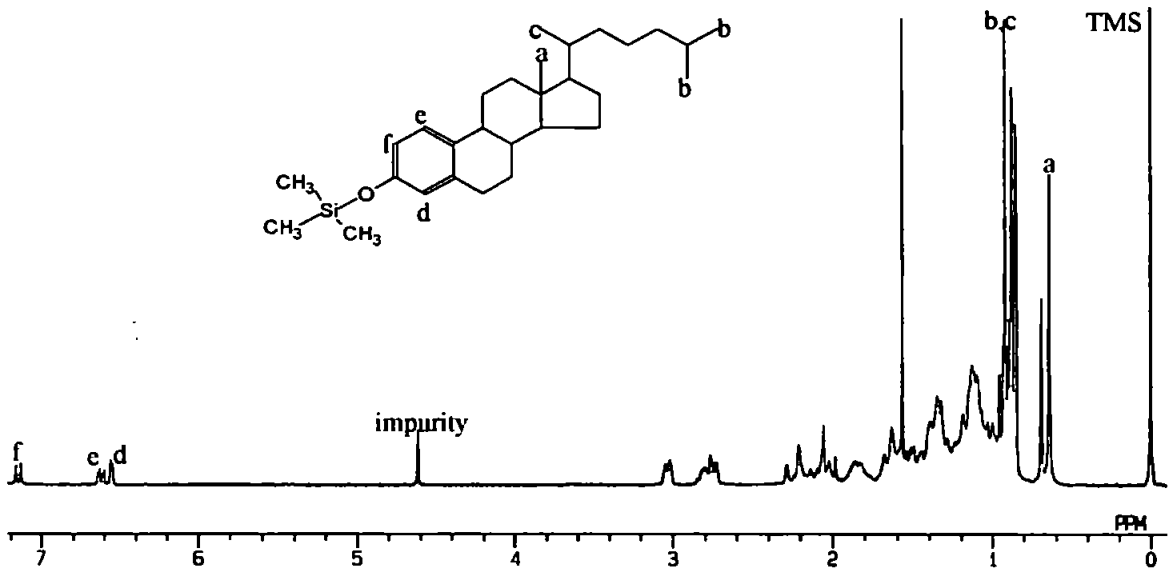
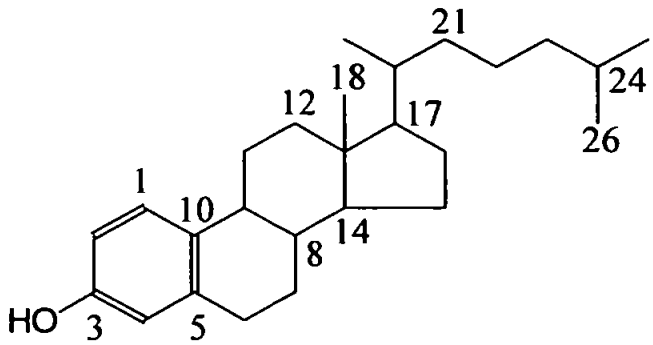


Figure 3.11 ^{1}H NMR spectrum of NCT TMS in $CDCl_3$ relative to TMS.

The NMR analysis was performed on the largest aliquot of NCT possible in order to obtain ^{1}H and ^{13}C spectra. However, the largest aliquot available (*ca* 40 mg) was only *ca* 85 % pure (determined by GC-MS) and thus the spectra obtained contained a degree on impurities. The total synthesis yield was *ca* 50 mg **LXXXI** of which 10 mg had a purity >97 % as determined by GC-MS of the TMS ether. This aliquot was used entirely to determine chromatographic properties and estrogenicity and thus detailed elemental analysis on the most pure fraction (by high resolution-mass spectrometry, HR-MS) was not possible.

The mass spectrum of NCT (**LXXXI**) TMS ether was determined by GC-MS for the first time (Figure 3.12) and comprised the expected molecular ion (*m/z* 440) as basepeak and significant but minor ions at *m/z* 368 (most likely due to underivatised **LXXXI**), *m/z* 285 and *m/z* 244. (Figure 3.12).



^{13}C NMR data for NCT (ppm) (present study)	Type of carbon Environment or carbon number	^{13}C NMR data from literature (ppm) (Kocovský and Baines, 1994)
11.99	CH_3	12.00
18.67	CH_3	18.69
22.55	CH_3	22.57
22.81	CH_3	22.83
23.79	CH_2	23.83
23.94	CH_2	23.94
26.78	CH_2	26.79
27.62	CH_2	27.66
28.02	CH	28.02
28.29	CH_2	28.30
29.71	CH_2	29.72
35.80	CH	35.81
36.16	CH_2	36.18
38.76	CH	38.78
39.52	CH_2	39.53
39.98	CH_2	39.95
42.79	C13	42.78
43.71	CH	43.71
55.42	CH	55.42
56.35	CH	56.36
112.51	C2	112.58
115.15	C4	115.20
126.49	C1	126.37
133.21	C10	132.88
138.00	C5	138.22
153.14	C3	153.29

Table 3.2 ^{13}C NMR obtained for NCT, compared with published data. The carbon environment or carbon number are also indicated.

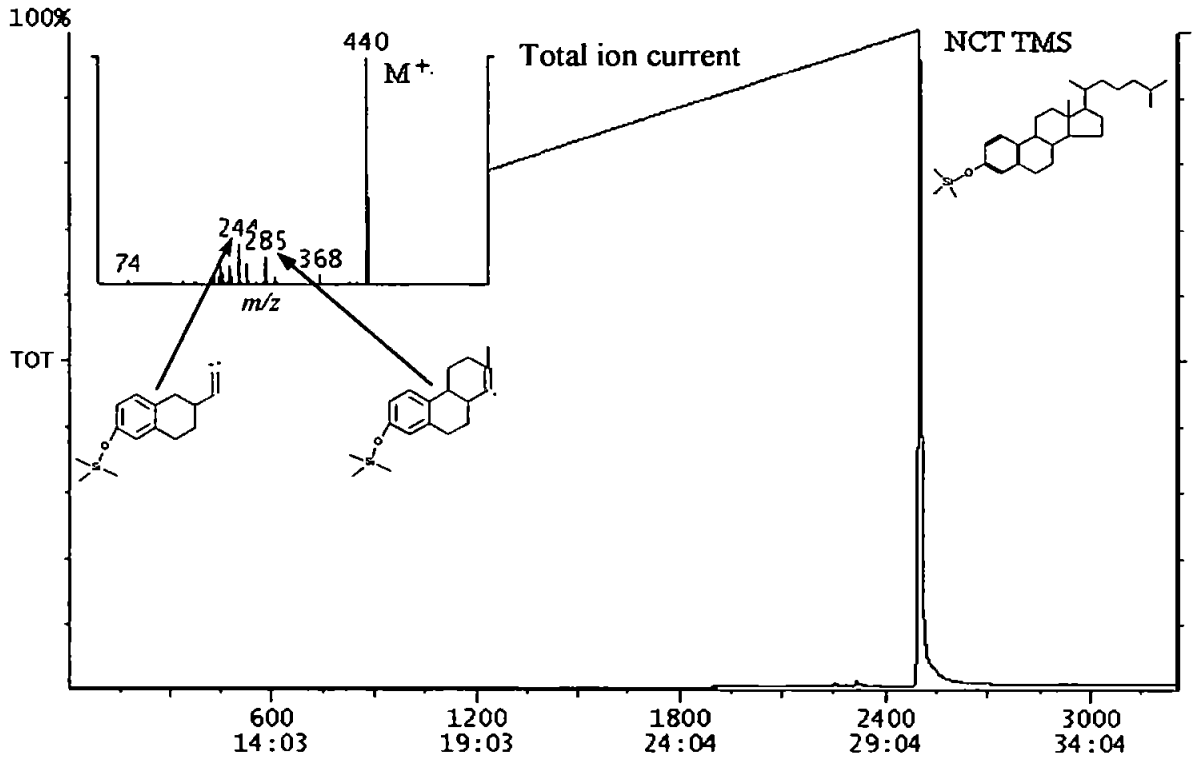


Figure 3.12 GC-MS total ion current chromatogram of purified aliquot of NCT (**LXXXI**) TMS ether (29:15 min) and the corresponding mass spectrum showing the $M^{+\cdot}$ as *m/z* 440.

Prof. J. Dearden, Liverpool John Moores University, computed a log K_{ow} value of 9.23 for NCT (**LXXXI**) using the Syracuse fragmentation algorithm indicating, as expected, that **LXXXI** is a very hydrophobic chemical despite the phenolic hydroxyl group.

3.4 Determination of NCT estrogenicity

The estrogenic potential of NCT (**LXXXI**) was assessed by the YES yeast assay (see Section 1.1.2, Chapter 1 page 3) and was kindly performed by Prof. J. Sumpter and colleagues of Brunel University, U.K. The result of the estrogenic test compared with a blank, cholesterol (**III**) and 17β-estradiol (**I**) is shown in Figure 3.13.

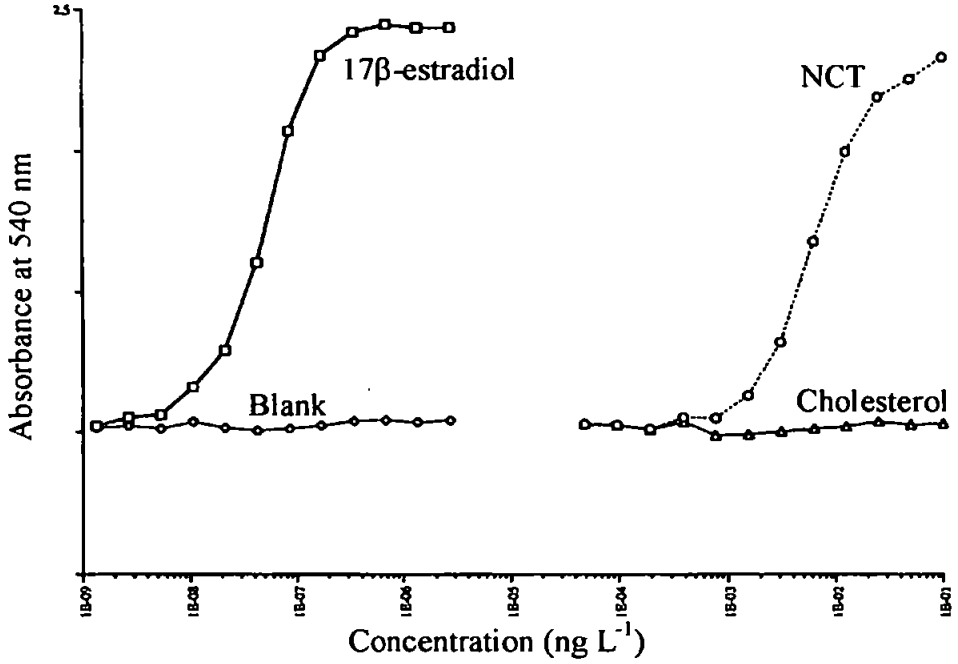


Figure 3.13 Estrogenic response of cholesterol (**III**) and NCT (**LXXXI**) by the Yeast Assay compared with a blank and 17β-estradiol (**I**).

The blank and cholesterol (**III**), as expected, showed no estrogenicity. The result of the estrogenic test for NCT (**LXXXI**) shows that **LXXXI** does have an estrogenic activity as defined by the YES assay. When comparing **LXXXI** with 17β-estradiol (**I**) (log K_{ow} of 4.01) the maximum estrogenic effect was determined to be 0.1 g L^{-1} for **LXXXI** compared with 2 x 10^{-6} g L^{-1} for **I**. Thus, **LXXXI** is *ca* 200 000 times less potent an estrogen than **I** when analysed by the yeast assay. The yeast assay of course assumes that the test compound **LXXXI** has been taken up by the yeast medium. However, with a theoretical log K_{ow} value for **LXXXI** >9 there is considerable doubt to the percentage of **LXXXI** uptake into the medium.

3.5 Discussion

Whilst the overall yield of NCT (**LXXXI**) synthesised herein (5%) was much lower than the 40 % reported by Afonso *et al.* (1966) by the same method, the **LXXXI** obtained herein was generally *ca* 85 % pure, however, a small 10 mg aliquot was >97 % (as determined by GC-MS of the TMS ether). The ^{1}H NMR spectrum of **LXXXI** (Figure 3.11) indicated that **LXXXI** was not pure, likely due to unused BSTFA, pyridine and solvents such as methanol still present (such impurities would not be visible by GC-MS). The synthetic procedure required the initial formation of the biphenyl-lithium electron donor prior to the addition of the dienone reactant. The reaction is susceptible to moisture and to be successful must be carried out under anoxic conditions. Cambie *et al.* (1969) attempted to prepare **LXXXI** using the conditions of Afonso *et al.* (1966) but were unsuccessful, producing only a non-phenolic oil. The authors reported that the biphenyl-lithium produced a red colouration and speculated it was due to the presence of moisture. Cambie *et al.* (1969) repeated the procedure and included diphenylmethane as described by Dryden *et al.* (1964). The initial colouration of the biphenyl-lithium was dark green, as observed herein, and yielded ~ 85 % **LXXXI**. The Afonso *et al.* (1966) procedure was in fact adapted from that of Dryden *et al.* (1964) eliminating the addition of diphenylmethane. Diphenylmethane was originally added to prevent the by-product, methyllithium, from adding to the carbonyl group at position C17 used by Dryden and co-workers to produce estrone (**IV**) (see Figure 3.1). The acid hydrocarbon, diphenylmethane, increased the yield of **IV** from *ca* 56 % to 75 %. Afonso and co-workers eliminated the need for diphenylmethane as the steroidal dienone starting material used had an alkyl side chain at position C17 rather than the more reactive carbonyl group used by

Dryden *et al.* (1964). The low yield reported in the current study is probably not due to the presence of moisture in the biphenyl-lithium since the expected dark green colouration was observed herein, but could be due to the presence of oxygen. The nitrogen used was ultra high purity (>99.99 %) but no oxygen scrubbing device was employed and hence it is speculated that the low yield could be due to small amounts of oxygen present. Although Cambie *et al.* (1969) included the diphenylmethane in their procedure it is unlikely that it was responsible for the high yield reported but most probably due to the enhanced formation of the biphenyl-lithium. In the present study, coprostan-3-one was also observed as one of the by-products of the reaction. If the reaction mechanism favours the reduction of the dienone rather than the A-ring aromatisation this could also explain, in part, the low yields obtained herein. The method of Dryden *et al.* (1964) was rather cumbersome and relies heavily on water and oxygen free environments. Kocovský and Baines (1994) describe a much simpler method of producing NCT (**LXXXI**) from 10β-hydroxy-19-norcholest-4-en-3-one (**LXXIII**) and possibly this would improve the yield of **LXXXI**. However, the starting material **LXXIII** was not readily available to the present author.

The computed log K_{ow} for NCT (**LXXXI**) of *ca* 9 suggests that if **LXXXI** were to be found naturally in the environment it would be associated with organic rich SPM. Thus, it is apparent that any examination of STW effluent for **LXXXI** should involve the examination of both the liquid and associated SPM.

Whilst the major aim of the **LXXXI** synthesis was to investigate whether **LXXXI** was an intermediate to estrone (**IV**) formation, the yeast estrogenic assay determined that **LXXXI** was also a weak estrogen, about 200 000 times less active than 17β-estradiol

(**I**). The use of the recombinant yeast strain containing the human estrogen receptor has been utilised on many natural compounds such as estrone (**IV**) and 17β-estradiol (**I**) and synthetic, xenoestrogens such as nonylphenol (**II**) (Routledge and Sumpter, 1996). The log K_{ow} values for these compounds range between 3-6 (the experimentally determined log K_{ow} value for **II** is 5.76, from Syracuse web site). The procedure requires serial dilution of individual compounds in ethanol to be added to the wells of a micro-plate and the ethanol evaporated off. The residual remaining had aliquots of assay medium added, shaken for 5 minutes prior to incubation. The procedure assumes that the compound under investigation is taken up into the assay medium. Compounds with relatively low log K_{ow} values such as **I** (4.01, experimentally determined, see Table 5.2) this is probably true, but as the log K_{ow} increases the degree of medium uptake is likely to decrease. Zhou *et al.* (1997) showed that ~ 70 % a hydrophobic insecticide, tefluthrin that has a similar log K_{ow} value as NCT (**LXXXI**), rapidly sorbed onto the glass wall of a reaction vessel rather than into solution. Thus the value of ~200 000 times less active than **I** is probably a minimum value and may not truly reflect the total estrogenic potential of **LXXXI**. Routledge and Sumpter (1996) comment that the yeast assay could give false negatives and suggest testing compounds with *in vivo* studies to determine possible metabolic by-products. **LXXXI** is known to be metabolised to **IV** by certain bacteria (Afonso *et al.*, 1966). There has not, as yet, been a study on mammalian and environmental metabolism of **LXXXI**.

3.6 Conclusion

NCT was synthesised, albeit at low yield, characterised by ^{1}H and ^{13}C NMR and the chromatographic and mass spectral properties of the TMS ether determined for the first time. These data will be valuable in studies of radiolabelled cholesterol metabolism

(Chapter 4) and for the monitoring of NCT (TMS ether) in wastewater effluents (Chapter 5). The estrogenic potential of the NCT was measured in an established bioassay. NCT was shown to have an estrogenic signature that was lower than that of 17β-estradiol but there is some doubt whether the yeast assay is reliable for the monitoring of very hydrophobic compounds. Other *in vitro* and *in vivo* assays should probably be used. The computed log K_{ow} value of 9.23 indicates that if NCT is to be found naturally in the environment as a metabolite of cholesterol biodegradation it would probably be associated largely with solid particulate matter (SPM) or other hydrophobic materials rather than in the aqueous matrix.

Chapter 4

Cholesterol metabolism during laboratory based sewage treatment

4.1 Introduction

Cholesterol (**III**) is the most abundant steroid in mammalian systems and is an important intermediate in the biosynthesis of sex hormones such as testosterone (**XVI**) and 17β-estradiol (**I**), (Fieser and Fieser, 1959). Mammals excrete **III** in high concentrations and the burden entering a STW ranges between 2 and 10 mg L^{-1} of sewage influent (Quéméneur and Marty, 1994). It is known that under the action of various bacteria **III** can undergo metabolism both aerobically and anaerobically yet the number of studies of biotic transformation of **III** in specialised environments such as sewage treatment is in fact still surprisingly small. Gaskell and Eglinton (1975) were two of the very few workers to examine the metabolism of radiolabelled **III** during incubation studies with anaerobic sewage sludge. In their studies coprostanol (**LXXII**) and cholestanol (**LXXXII**) were identified as the major products of cholesterol metabolism. Minor products were cholest-4-en-3-one (**LVII**) and 5α and 5β saturated keto cholesterols.

In contrast, many authors have examined cholesterol (**III**) transformation by single bacterial cultures (Charney and Herzog, 1967; extensive review of steroidal biotransformation). For example, Harvath and Kramli (1947) used cultured *Azotobacter* with **III** as carbon source and identified cholest-4-en-3-one (**LVII**) and cholest-5,7-dien-3β-ol (**LXVIII**) as major metabolites. The authors later identified cholest-5-en-7-one-3β-ol (**LX**) as a cholesterol (**III**) metabolite using the bacterium *Proactinomyces roseus* (Harvath and Kramli, 1948, 1949). The biodegradation of **III**

has been shown, in the main, to occur at two specific sites on the **III** molecule, *viz.* at the A/B-ring and at the C17 alkyl side chain. Sih and co-workers extensively studied the microbial mediative oxidative cleavage of the alkyl side chain of **III** (Sih *et al.*, 1967a,b) with a suite of bacterial cultures. The microbial degradation of **III** A-ring has been shown to be in competition with B-ring cleavage. Dobson and Muir (1961) showed that A-ring aromatisation of androst-1,4-dien-3,17-dione (**L**) occurred after the B-ring had been opened (between C9 and C10) forming 3-hydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (**LXVIII**). Sih *et al.* (1965a) showed that **LXVIII** could undergo further metabolism *via* the further hydroxylation at position 4 to produce 3,4-dihydroxy-9,10-secoandrosta-1,3,5-trien-9,17-dione (**LXIX**). The 4-hydroxyl group on the aromatic A-ring of **LXIX** allowed oxidative cleavage of the A-ring (*i.e.* C4-C5 bond cleavage) to produce 3aα-H-4α-[3'-propionic acid]-7aβ-methyl-hexahydro-1,5-indandione (**LXX**).

In the present study the metabolism of cholesterol (**III**) was examined during laboratory-based aerobic and anaerobic sewage treatment. ^{14}C-cholesterols radiolabelled at position 4 on the A-ring and position 26, an angular methyl on the alkyl chain were used to identify aerobic metabolites of A-ring and side chain oxidation respectively.4-^{14}C **III** only was added to activated sludge to study anaerobic biodegradation. The use of two separately radiolabelled cholesterols under aerobic conditions meant that a fuller analysis of **III** metabolism during wastewater treatment was possible than heretofore.

4.2 Anaerobic metabolism of cholesterol

On average 280 mg DW, anaerobic activated sludge was added to 27 serum vials and made up to 100 mL with water. The vials were sealed and allowed to become anaerobic prior to the addition of 4-^{14}C cholesterol (**III**) 4.9 KBq sample^{-1} (equivalent to 1μg L^{-1} **III**). Bottles were incubated at ~30 °C for a maximum length of 9 weeks. Each week three samples were sterilised with the addition of $HgCl_2$ and removed from the incubator. When required for analysis the samples were centrifuged and the supernatant separated from the solid fraction and retained. The solid fractions were extracted by ultrasonication with methanol (x 4) and extracts examined by LSC. Results for the solid samples showed a little variability in radioactivity over the 9-week period (Figure 4.1). The liquid supernatant samples were extracted by SPE and eluted into methanol. Eluants were then examined by LSC and contained on average 18 % ± 3, n = 5, of the total activity. Of the total activity only *ca* 6 % was retained by the SPE cartridge, *ca* 12 % remaining in the aqueous fraction.

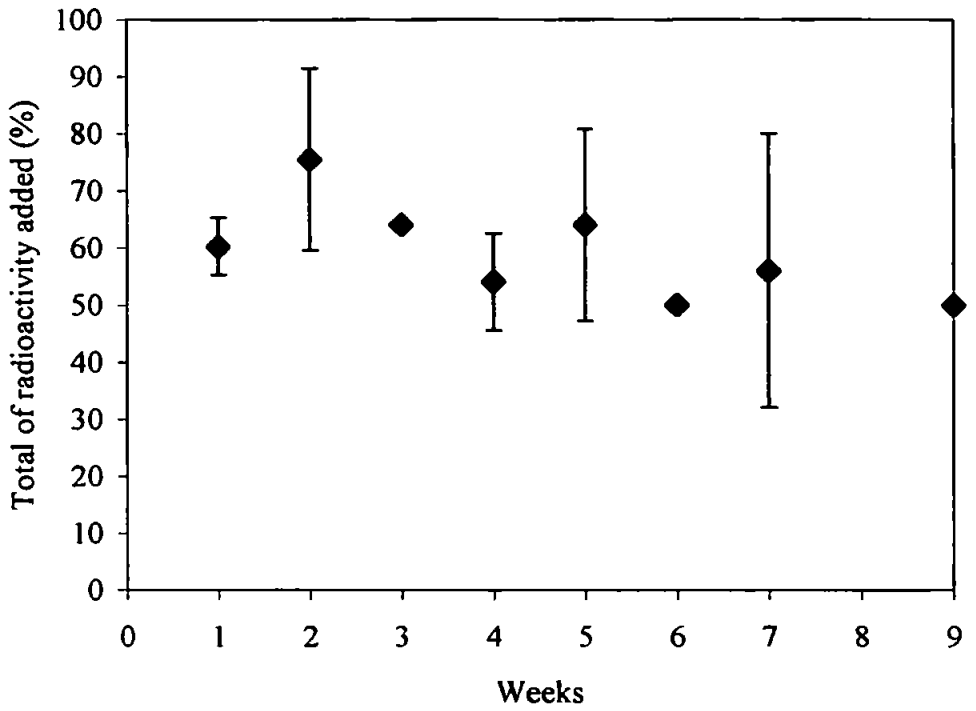


Figure 4.1 Percentage activity and variability determined in solid portion of anaerobic sludge after incubation of 4-^{14}C cholesterol over the 1 to 9 week incubation period. Error bars show ± 1σ (n = 3).

Extracts of both the solids and supernatant samples resulting from the laboratory incubations were fractionated by radio HPLC (rHPLC). $^{14}CO_2$ and other gaseous ^{14}C (*e.g.* $^{14}CH_4$) evolution was not determined in the samples.

The retention time for unincubated 4-^{14}C cholesterol (**III**) was 40 ± 2 minutes (n = 5). The efficiency of the rHPLC detector was *ca* 20 %. Examination of the supernatant samples revealed that all were <LOD (*i.e.* < 7 cps) for the detector. Nonetheless rHPLC fractions were taken at 5 minute intervals and retained for radio GC (rGC) analysis. No radioactive metabolites were determined for the supernatant samples. The rHPLC extracts of the solids, which contained most of the radioactivity (Figure 4.2 and

4.3), revealed two components which eluted between 37 and 40 minutes (Figure 4.2). The first component, RT 38:20 minutes coincide with the RT for NCT (**LXXXI**), whereas the second component, RT 39:00 minutes, was cholesterol (**III**). A small number of samples also appeared to contain minor radiolabelled components with rHPLC retention times of ~3-4 minutes. These compounds were not examined further but may be short chain polar compounds resulting from A-ring opening at C-4.

The radioactive HPLC fractions (37-42 minutes) of the solid extracts were retained, blown down to dryness under a constant stream of nitrogen and a thick yellow solid obtained. The samples were analysed by rGC as the TMS ether derivatives. DCM was used to dilute the samples. However, the volume was kept to a minimum (~ 10 μL) in an attempt to increase radioactivity introduced to the rGC. The compounds eluting from rGC column were detected by FID and RD. The split ratio between the detectors was heavily biased (1:9) to the RD. The efficiency of the RD was higher than that of the rHPLC (45 % - 85 %). The lag time between the FID and RD ranged from 8 to 20 seconds. Initially 10 % of the sample extract was analysed by rGC. This gave a strong FID response but the radioactivity signal was <LOD for all samples (Figure 4.3). The RD/FID split ratio was increased so all the sample was diverted to the RD and sample volume increased to 20 % of the total volume. However, no radioactive peaks were detected. This suggests that either the rGC method was too insensitive to detect the metabolites revealed by rHPLC (Figure 4.1) or that the metabolites were not amenable to GC analysis (*e.g.* were possibly polar components). The latter seems unlikely, however, given the rHPLC retention characteristics of the metabolites (*viz* close to that of cholesterol).

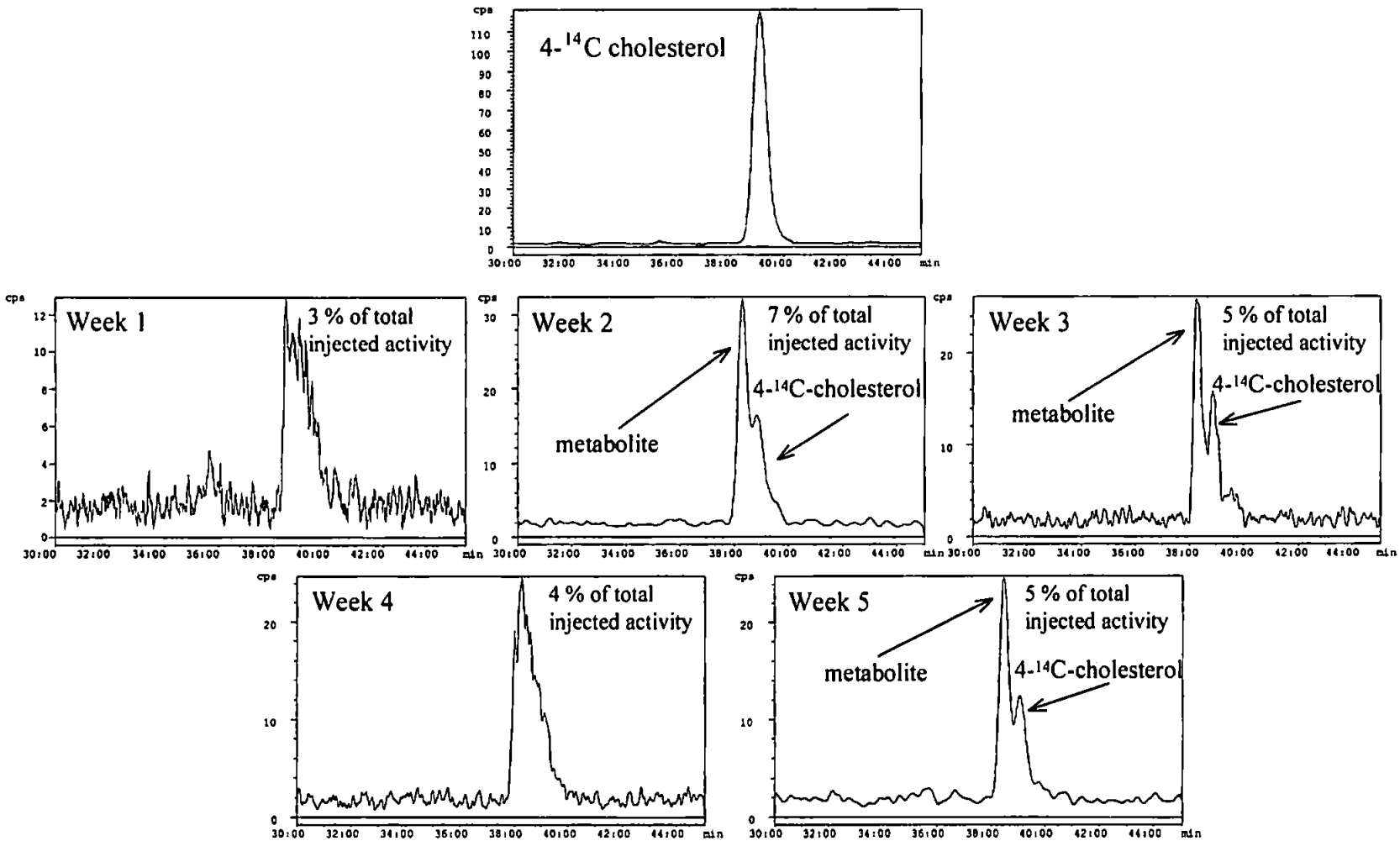


Figure 4.2 Chromatography results from rHPLC fractionation of methanolic extracts of solids after incubation of 4-^{14}C-cholesterol (**III**) with anaerobic activated sludge over 5 weeks under anaerobic conditions. The appearance of at least one new metabolite of 4-^{14}C **III** is obvious.

Methods of increasing the proportion of radioactivity in the incubation samples were investigated, principally by further fractionation of the extracts.

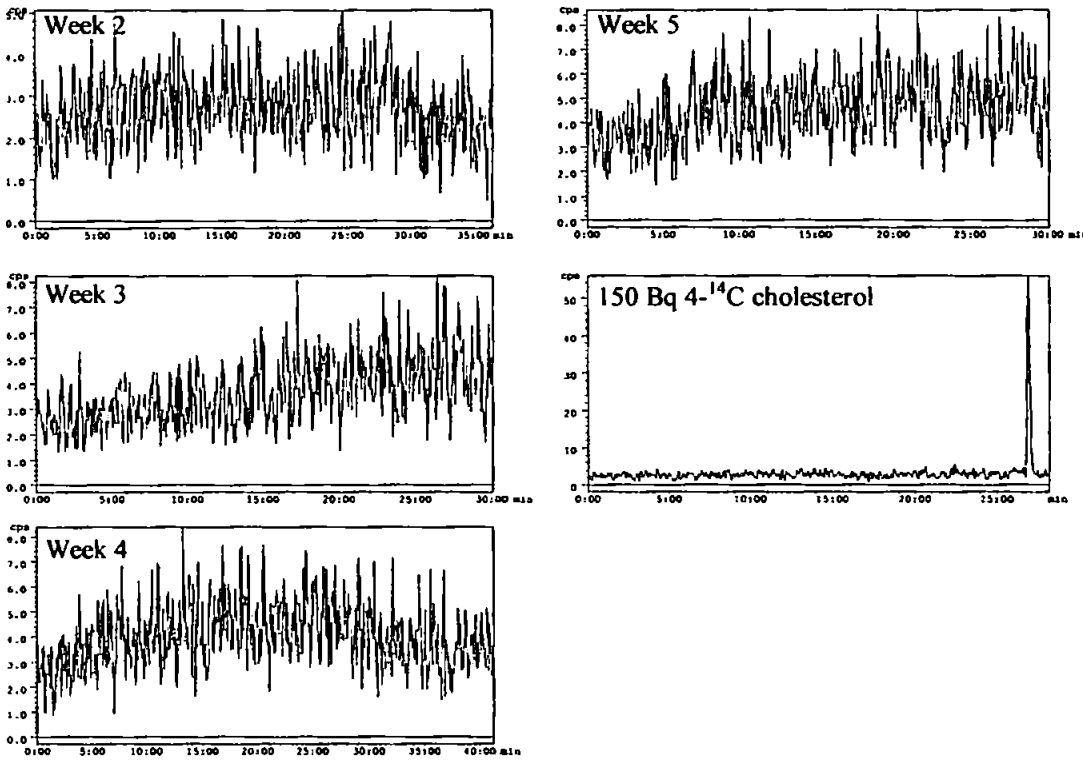


Figure 4.3 rGC analysis (RD only shown) of anaerobic solids extracts incubated 4-^{14}C cholesterol (**III**). The extracts were previously fractionated by rHPLC (fractions collected between 37-42 minutes, Figure 4.2). For comparison 4-^{14}C **III** was also analysed.

In an attempt to increase radioactivity and to decrease the unlabelled steroids and other components in the rHPLC fractions radio TLC (rTLC) was employed, prior to rHPLC in repeat incubation experiments.

The efficiency of the rTLC was determined to be ~11 % (peak height (CPS)/true activity (DPS)). The incubated solid anaerobic sludge samples were extracted and initially chromatographed on a thin layer of silica using DCM as the mobile phase. This resulted in the detection of two radioactive peaks (Figure 4.4). Both peaks a and b (peak b coincided with R_f 0.37 for NCT) of the chromatogram of solids extracts from weeks 4 and 9 were isolated by extraction into methanol and the radioactive component with, R_f 0.25-0.4, was re-chromatographed over silica using acetone:hexane (1:1) (Figure 4.5).

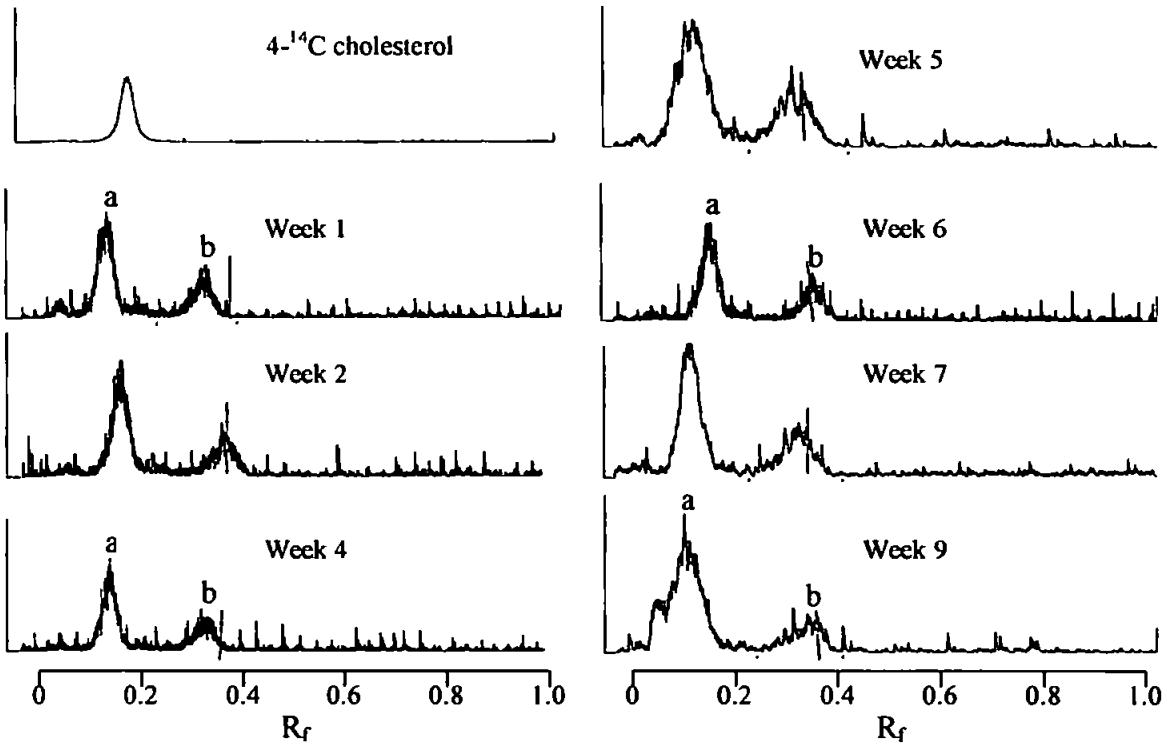


Figure 4.4 rTLC separation of anaerobic solid extract using DCM over silica and showing relative R_f values of compounds of interest. The relative R_f for NCT is shown as a red line.

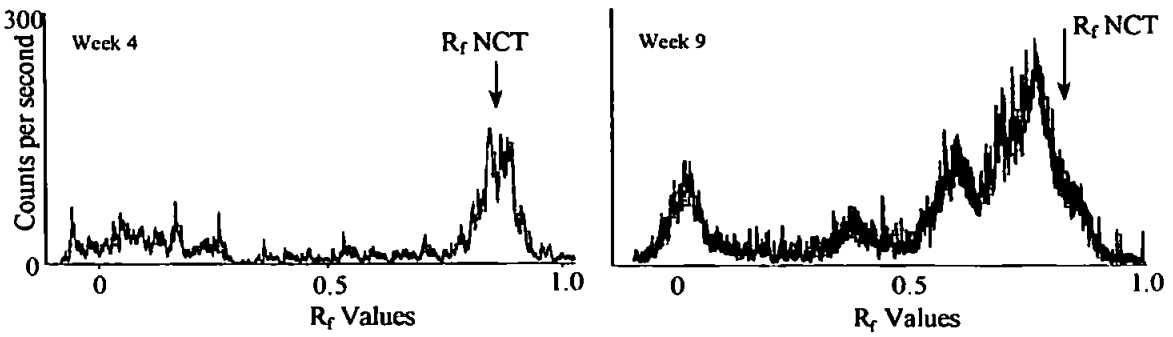


Figure 4.5 rTLC separation of rTLC peak b (R_f 0.25-0.4) in Figure 4.4 using hexane:acetone (1:1) over silica. The R_f value for NCT is shown.

The R_f value for NCT (**LXXXI**) using hexane:acetone (1:1) over silica was 0.83-0.97, which coincided with the radioactive region shown in Figure 4.5. The radioactive region was re-extracted for analysis by rGC as the TMS ether derivative (Figure 4.6).

Despite these further fractionation steps and the high concentration of extract injected into the r-gas chromatograph, few rGC components were observed in any of the extracts of either solid or supernatant. Figure 4.6(a,c) shows a partial rGC chromatogram in the retention time range of the expected steroid metabolites. On the basis of the rGC there was no evidence to suggest that radiolabelled NCT (**LXXXI**) was produced, despite the coincidence of the rTLC peak (Figure 4.5) in hexane/acetone with the R_f of synthetic **LXXXI**. The first radioactive peak 'a' (R_f 0.15) detected by rTLC on silica/DCM (Figure 4.4) was also analysed by rGC, however, no peaks were observed in the RD chromatogram. The major unlabelled components of both silica/DCM rTLC fractions (Figure 4.4) determined by GC-MS and proved to be steroidal alcohols (peak a, R_f 0.15) and ketones (peak b, R_f 0.25-0.4) (Figure 4.7).

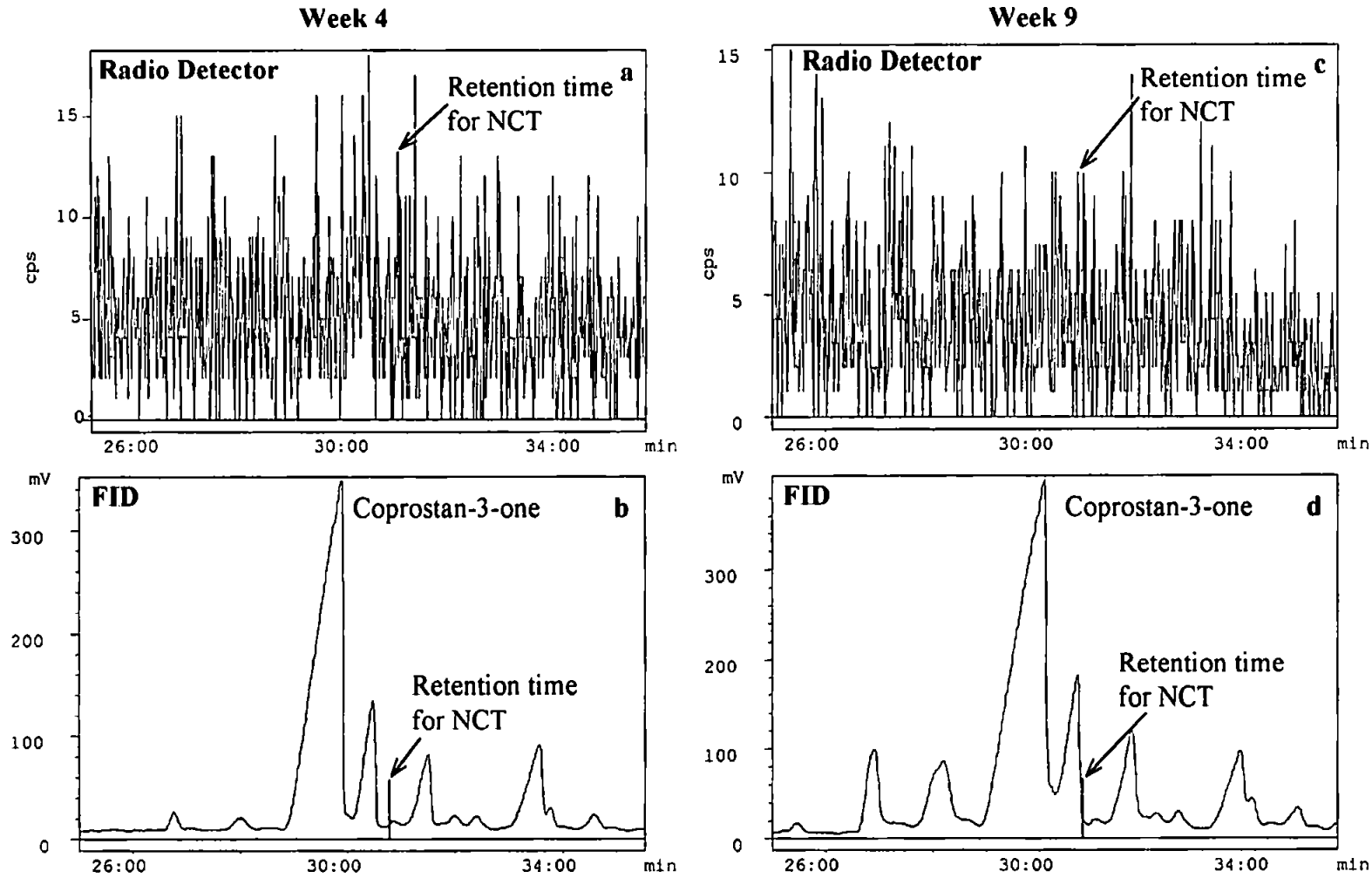


Figure 4.6 rGC (coupled with FID) of the radioactive rTLC peak obtained in Figure 4.5. Coprostan-3-one was identified in the FID chromatogram. The retention time of NCT TMS is also highlighted. It is clear that in these samples at least NCT, the putative cholesterol to estrone intermediate was not produced in detectable quantities.

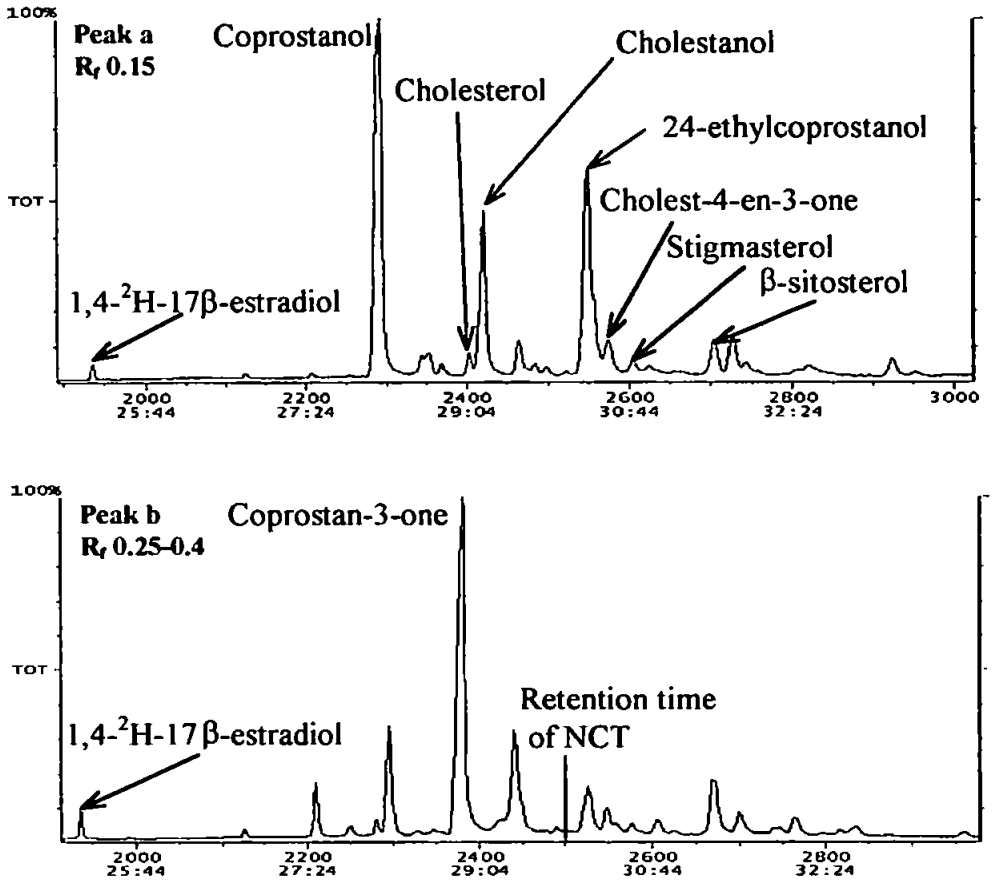


Figure 4.7 Partial GC-MS total ion current chromatogram of radioactive peaks a and b (R_f 0.15, 0.25-0.4) determined by rTLC (DCM/silica) (Figure 4.4) as the TMS ether derivatives.

Fraction R_f 0.15 contained the steroids such as coprostanol (**LXXII**), cholesterol (**III**) and β-sitosterol (**XXXI**) whereas fraction R_f 0.25-0.4 contained mainly coprostan-3-one (**LXXXIII**). NCT (**LXXXI**) was not determined in any radioactive fraction from any of the incubated anaerobic samples (weeks 1-9).

In all, 18 of the 27 (9 triplicates) anaerobic samples incubated over the 9-week period were analysed by rTLC and resulting fractions obtained were analysed by rGC.

However, no further information was gained. Whilst rHPLC and rTLC indicated at least one major product of anaerobic biodegradation, this compound(s) could not be identified despite extensive rGC analysis. Certainly and importantly, NCT (**LXXXI**), a proposed intermediate of cholesterol to estrone conversion, was not present in any of the fractions.

4.3 Aerobic metabolism of cholesterol in simulated sewage treatment

4.3.1 Semi-continuous activated sludge method (SCAS)

Four semi-continuous activated sludge (SCAS) units were used to model the aerobic microbial metabolism of cholesterol (**III**) (see Figure 2.2 page 62, for a diagram of a SCAS unit). SCAS system is widely accepted as a means of simulating continuous flow activated sludge systems of domestic sewage treatment works (STW). The SCAS medium contained ~0.6 g of activated sludge made up to 1 L with tap water. Aeration to the SCAS system (CO_2 free laboratory air) was stopped once every 23 h and 100 mL of mixed liquor (liquid containing solids) and 400 mL of liquid (solids settled out) were removed for analysis. Before aeration was restarted the system was made up to 1000 mL with the addition of fresh sewage influent. Therefore, unlike a full-scale domestic STW, the microorganisms in a SCAS system were not replenished by the continuous addition of sewage influent and hence a true dynamic representation of the processes that occur in an activated sludge STW is not achieved. Nonetheless the cholesterol content of the activated sludge and influent in the SCAS was determined in order that the amounts of radioactive cholesterol (**III**) added to the system would not dramatically alter the overall **III** content. The solids sludge contained 13 ± 2 mg g^{-1} (dry weight of solid) compared with 40 ± 5 μg L^{-1} for the influent.

The units were housed in a temperature controlled laboratory (~20° C) under controlled artificial lighting in order to maximise bacterial growth and to minimise algal production. The units were aerated with CO_2-free air. Before entering the SCAS the air was passed through a 2M solution of NaOH to trap atmospheric CO_2. Air was drawn through the system under negative pressure. Two NaOH traps were employed, inline, to trap $^{14}CO_2$. Prior to the addition of radiolabelled cholesterol (**III**) the SCAS unit was aerated and sub-samples taken to determine total biomass. When the system reached optimum performance (*i.e.* as indicated by a stabilisation of biomass determined gravimetrically) 49 KBq (equivalent to ~10 μg) 4-^{14}C III was added. The natural **III** content in the SCAS liquor was >10 mg L^{-1} and hence the addition of the radiolabel contributed *ca* 0.1 % **III**. It is important that ^{14}C addition does not perturb the system by addition of excessive mass of substrate. Clearly this has been avoided in the present study (Dr. J. Snape, *personal communication*).

The SCAS units were aerated for 23 h, after which time 100 mL of mixed liquor (10 % of total) was removed for total biomass (25 mL filtered through a GFC filter for total biomass activity determination) and the remainder (7.5 % of total biomass) was solvent extracted for chromatographic studies. The remaining 900 mL of liquor in the unit was allowed to settle over a period of 1 h and 400 mL of supernatant drawn off for SPE. Fresh influent was added to top up the SCAS unit and synthetic feed and aeration re-applied. Combustion of solid fractions showed that after 15 minutes 78 % of the cholesterol (**III**) added was associated with the particulate matter. To determine mineralisation rates the NaOH CO_2 traps were replaced daily and the activity determined by LSC (Figure 4.8).

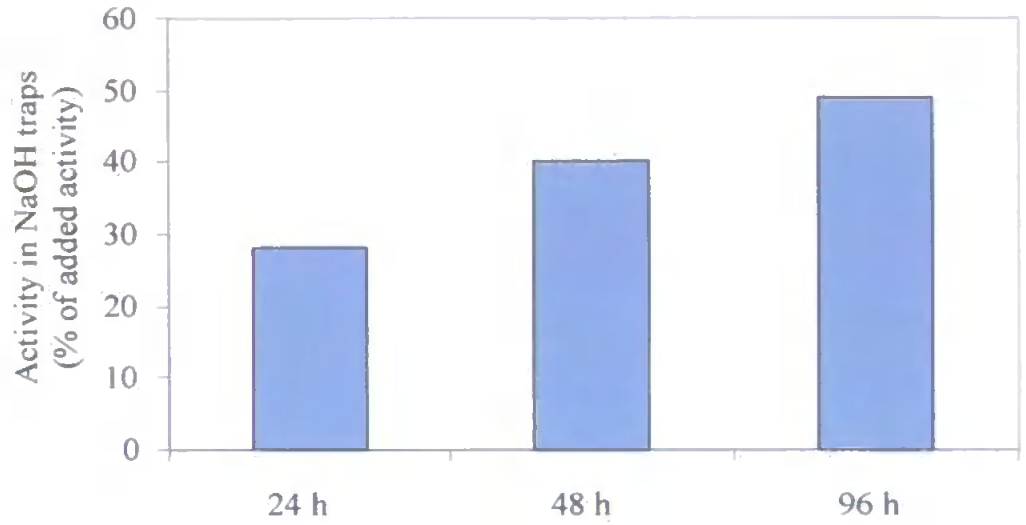


Figure 4.8 Amount of $^{14}CO_2$ evolved from 4-^{14}C-**III** during aerobic SCAS incubation. The amount of $^{14}CO_2$ produced is represented as a percentage of total radiolabelled **III** originally added (n = 1).

The NaOH traps did not contain any volatile radiolabelled species when analysed by LSC (Dr. J. Snape, *personal communication*) but showed that > 25 % of the total radiolabel added had been mineralised to CO_2 in the first 24 h and *ca* 50 % after 96 h (Figure 4.8). The relative proportions of radioactivity determined from the mixed liquor from the 24 h – 96 h incubations are shown in Figure 4.9.

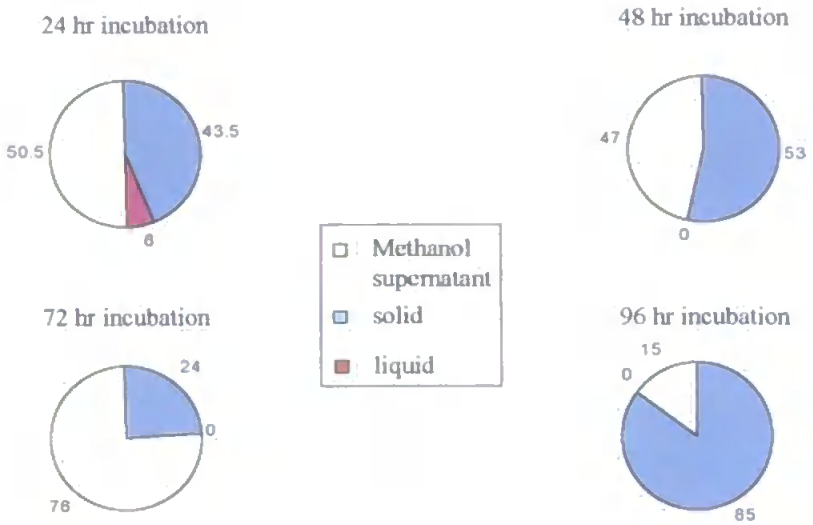


Figure 4.9 The percent activity of 24, 48, 72 and 96 h incubations in solids, solid supernatants (50 % methanol added to solid fraction during transportation of sample as microbial inhibitor) and aqueous liquid fractions from the mixed liquor.

The radioactivity in the mixed liquor was significantly biased to the solid fraction (including methanol supernatant). Radioactivity in the aqueous fraction accounted for just 6 % of the total radioactivity in the first 24 h. No radioactivity was detected in the aqueous fraction after 24 h.

The liquid extracts obtained from the solid phases, including the 50 % methanol supernatant of all the samples, were fractionated by rHPLC (Figure 4.10). Apart from unreacted cholesterol (24 h) and some minor polar compounds, no metabolites were detected in the SPE extracts of the aqueous phase. Even these minor products were absent by 48 h (Figures 4.9 and 4.10).

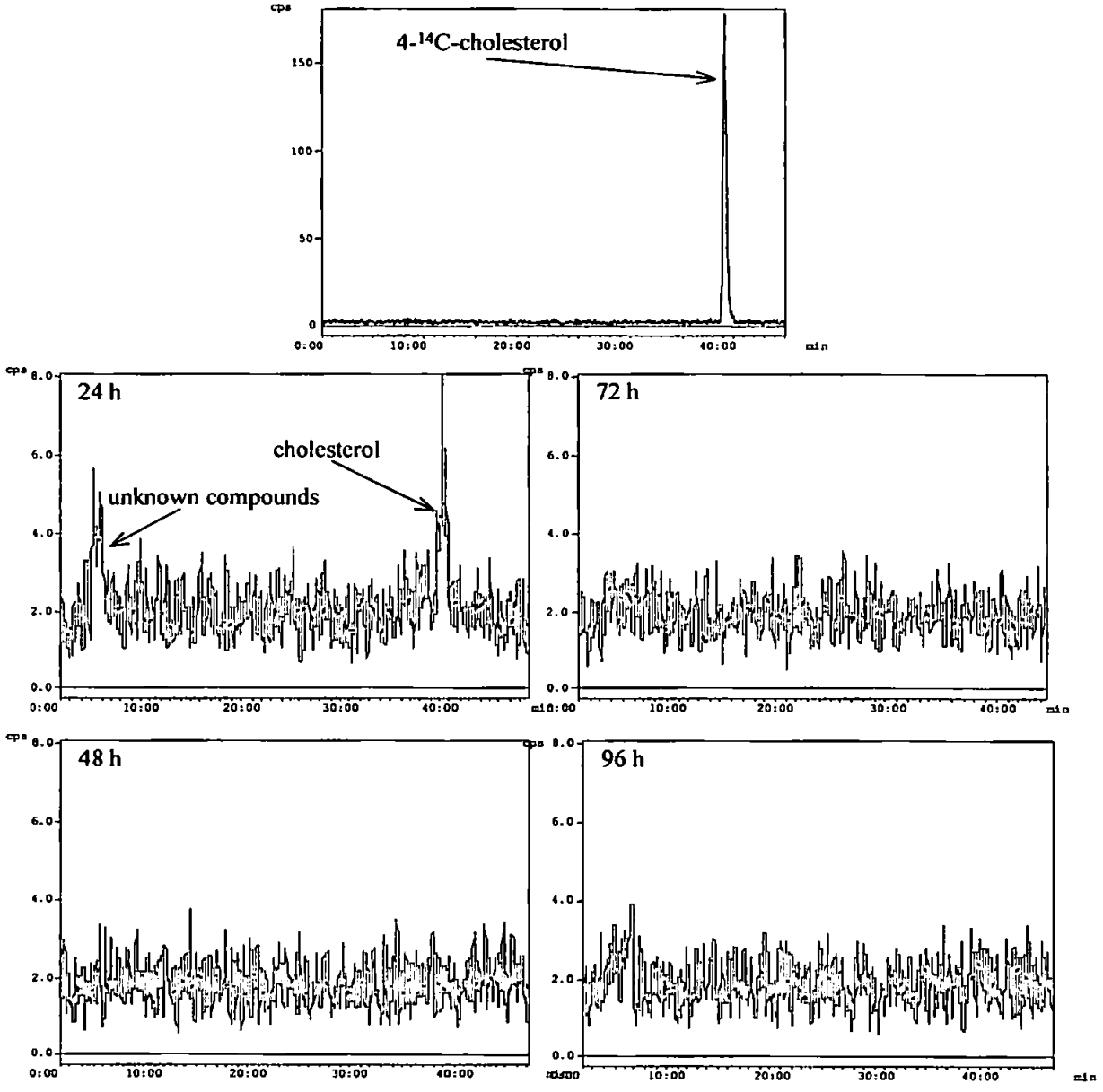


Figure 4.10 rHPLC fractionation of SPE extracted liquid samples incubated aerobically in a SCAS system over 96 h. The occurrence of cholesterol (**III**) (38:12 minutes) and unknown early eluting compounds (3:16 minutes) after 24 h are visible.

Radiolabelled HPLC fractions between 0-5 and 38-42 minutes were collected and examined by rGC but the activity was <LOD (*i.e.* <7 cps) for the RD.

Extracts from the solid phases (*ca* 3 – 5 % of total injected activity) from all time series samples were analysed by rHPLC and two peaks were consistently observed (Figure 4.11). The largest peak eluting at *ca* 38 minutes coincided with 4-^{14}C-cholesterol (**III**).

The unknown compound shown in Figure 4.11 occurred in all samples and represented 16-29 % of the total activity within the solid extracts and 8 - 19 % of total injected activity (Table 4.1).

	Incubation Time (h)			
	24 h	48 h	72 h	96 h
Total activity in solid sample (KBq) (in 7.5 % of total biomass)	2.50	1.96	1.54	1.24
Activity of unknown metabolite (% of total activity in the sample)	29	22	16	18
Activity of unknown metabolite (% of total injected activity)	20	12	7	6

Table 4.1 The occurrence and percentage of unknown metabolite for all aerobic SCAS solid extracted samples (24 – 96 h).

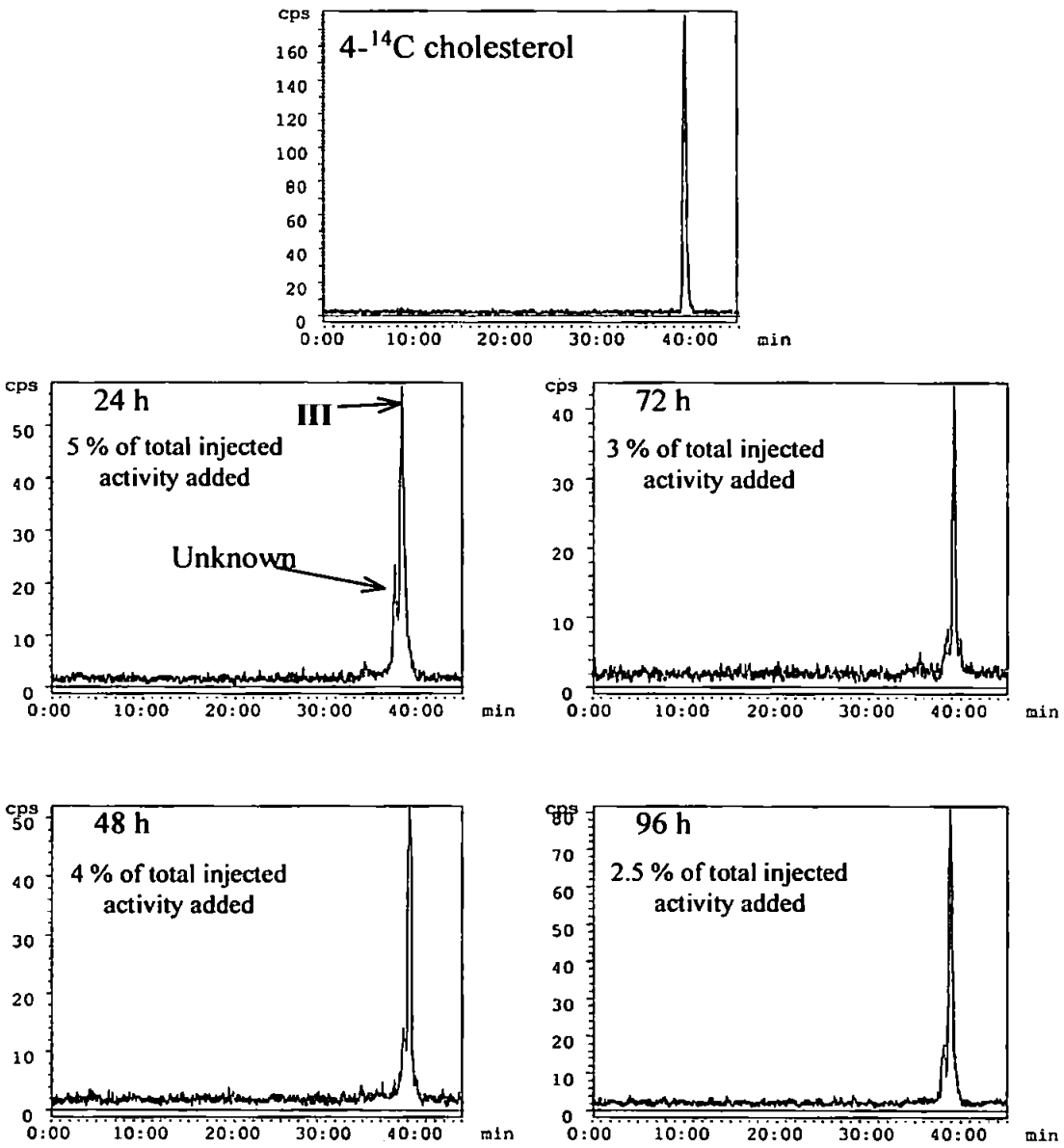


Figure 4.11 rHPLC analysis of extracts of solids (7.5 % of total biomass) incubated aerobically in a SCAS system over the course of 96 h. The chromatograms clearly show that the majority of radioactivity was due to non metabolised cholesterol (**III**), however, an unknown metabolites was present in all samples fractionated.

Fractions containing both rHPLC peaks, (35-42 minutes) were isolated and analysed by rGC. Cholesterol (**III**) was identifiable in all fractions (Figure 4.12), however, an unidentified metabolite was observed in the fraction from 48 h incubation.

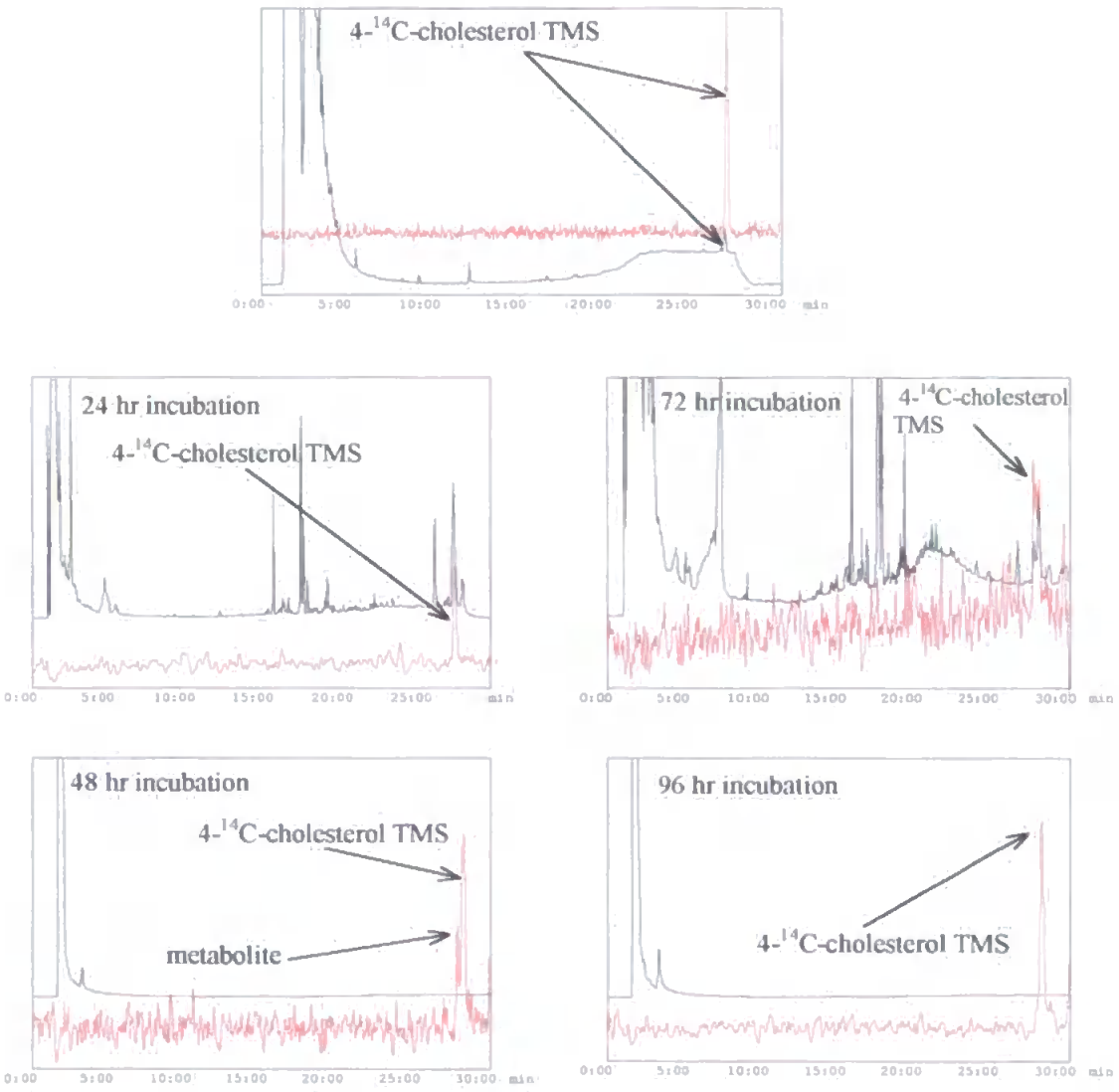


Figure 4.12 rGC analysis of rHPLC fractions taken between 35-42 minutes (Figure 4.11) over the 96 h aerobic incubation peroid. Cholesterol (**III**) can be clearly seen as the dominant radioactive component in all extracts. The black line represents the FID output and the red line represents the signal from the RD.

The use of the SCAS system to study the aerobic biodegradation of 4-^{14}C cholesterol (**III**) showed that the majority of activity in the mixed liquor was associated with SPM (*ca* 95 %). The activity in the liquid fraction was either due to **III** or short chain polar compounds. The mineralisation rate was rapid, 29 % in the first 24 h, yielding $^{14}CO_2$. Thus, the A-ring of **III** had been ruptured leading to the subsequent release of the 4-^{14}C atom. Prior to $^{14}CO_2$ production the 4-^{14}C atom was most probably incorporated into the short chain organic acids which were tentatively identified in the liquid fractions. The removal of the 4-^{14}C atom almost certainly resulted in further unlabelled biodegradation products which could not be detected by radio methods. The SPM sample extracts contained two compounds >LOD when fractionated by rHPLC. For all samples examined **III** was the major component. Not surprisingly the minor metabolite was not detected by rGC. The minor metabolite could be possibly be an isomer of **III** which co-eluted with **III** when analysed by GC. The metabolite seen in 48 h incubation fraction was not identified.

4.3.2 Die away method

The so-called "die away" (DA) method for assessing the metabolism of cholesterol (**III**) is a simpler version of the SCAS system previously described (see Figure 2.3, page 64, for a diagram of a DA unit). Fresh sewage influent (1 L) containing 26-^{14}C **III** was aerated with CO_2-free air. $^{14}CO_2$ evolved due to mineralisation was trapped into two in-line NaOH traps. The micro-organisms present in the influent were not given synthetic feed or replenished with daily additions of fresh influent. In the present study the DA method was used to examine **III** metabolism over 24 and 48 h. An abiotic control experiment was also carried out to determine non-microbial breakdown

processes. 26-^{14}C **III** was used in an attempt to further identify some of the products of A-ring opening which had been shown to occur in the SCAS experiment.

In total, three units were run simultaneously (24 h, 48 h metabolism and time zero blank). To each DA vessel containing sewage influent, 740 KBq of radiolabelled cholesterol (**III**) was added and the mixture allowed to aerate. To the time zero blank 70 mg of mercuric chloride was added as a bacterial inhibitor prior to the addition of the radiolabel and the whole allowed to aerate for 30 min. The abiotic control was aerated for a period of 24 h. The two NaOH traps for all three units were analysed for $^{14}CO_2$ activity by LSC (Figure 4.13).

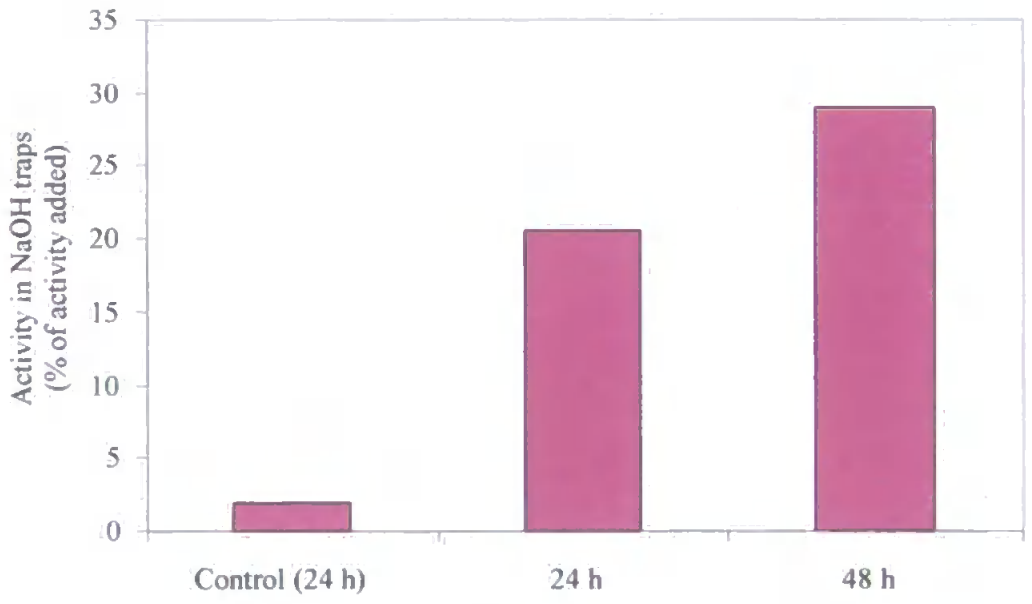


Figure 4.13 Amount of $^{14}CO_2$ evolved as a percentage of total 26-^{14}C **III** added to Die Away aerobic sewage incubation system (n = 1).

The amount of $^{14}CO_2$ evolved from the time zero blank (1.9 %) was much lower than amount produced for 24 h (21 %) and 48 h (29 %) incubations. These results show

that by far the majority of 26-^{14}C **III** mineralisation was biologically mediated and that mineralisation increased over time.

When aeration was ceased the liquor in each DA unit was divided into 4 x 250 mL and the solids separated from liquids by centrifugation. The amount of solid material present was *ca* 2 g L^{-1}. In order to quench any further microbial activity, prior to extraction, the solid samples were immediately stored at -20° C. The supernatant samples were immediately extracted by SPE. Aliquot samples of the supernatant before and after SPE, determined by LSC, showed that *ca* 50 % was retained by the cartridge (Figure 4.14).

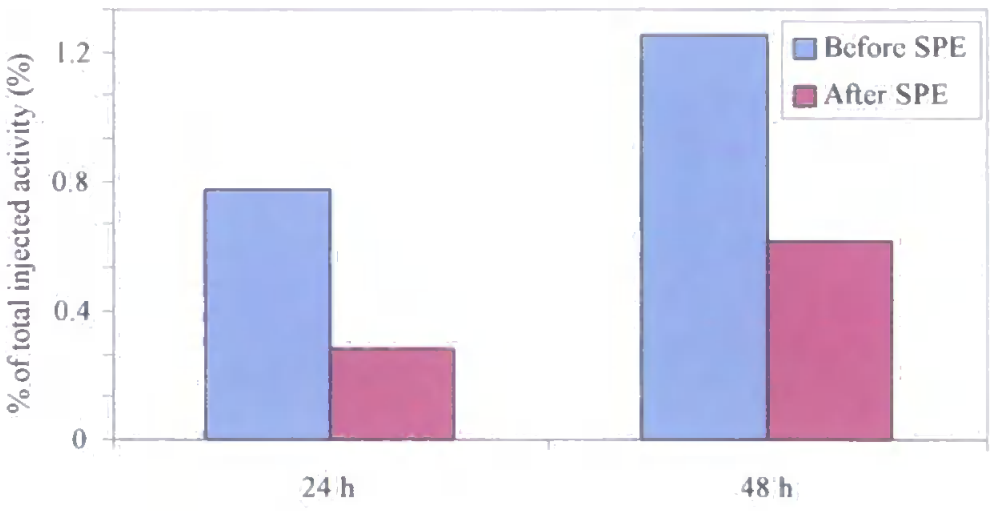


Figure 4.14 Radioactivity in the supernatant, as a percentage of total injected activity, from the Die Away system before and after SPE. The SPE cartridge retained *ca* 50 % of the activity present in the supernatant.

Re-extraction of the supernatant samples by SPE resulted in no further increase in retention of radioactivity.

When required, solid material was defrosted and extracted into methanol. Aliquots of the extract were taken for LSC determination. The solid fractions contained the majority of activity, *ca* 80 %. The mineralisation and subsequent $^{14}CO_2$ production accounted for almost all-remaining activity with only *ca* 1 % dissolved in the aqueous portion (Figure 4.15).

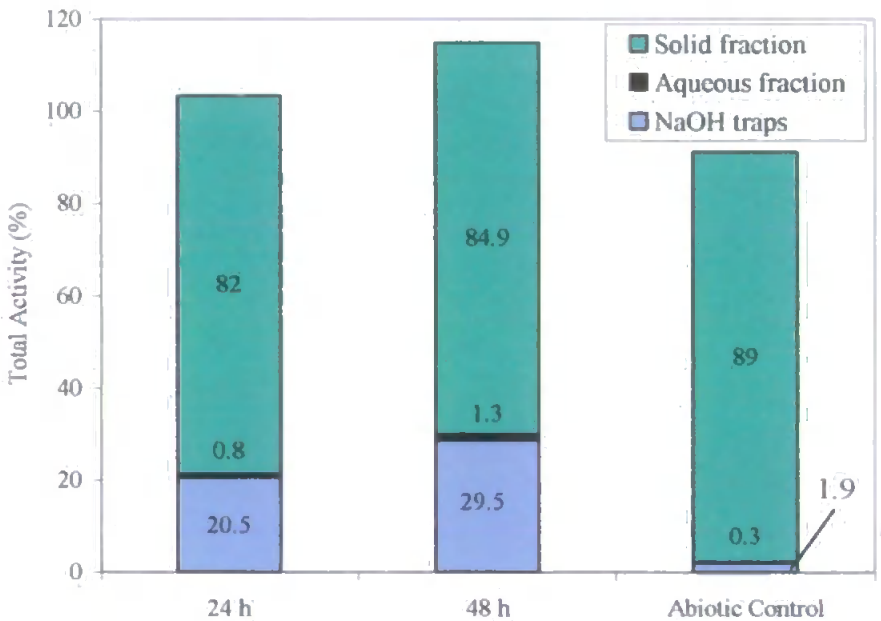


Figure 4.15 Mass balance for 24, 48 h and abiotic control Die Away incubations of 26-^{14}C cholesterol (**III**).

The rHPLC fractionation of the SPE extracts of the liquid phase showed that unidentified polar compounds were the major products present in addition to a small amount of **III** (Figure 4.16).

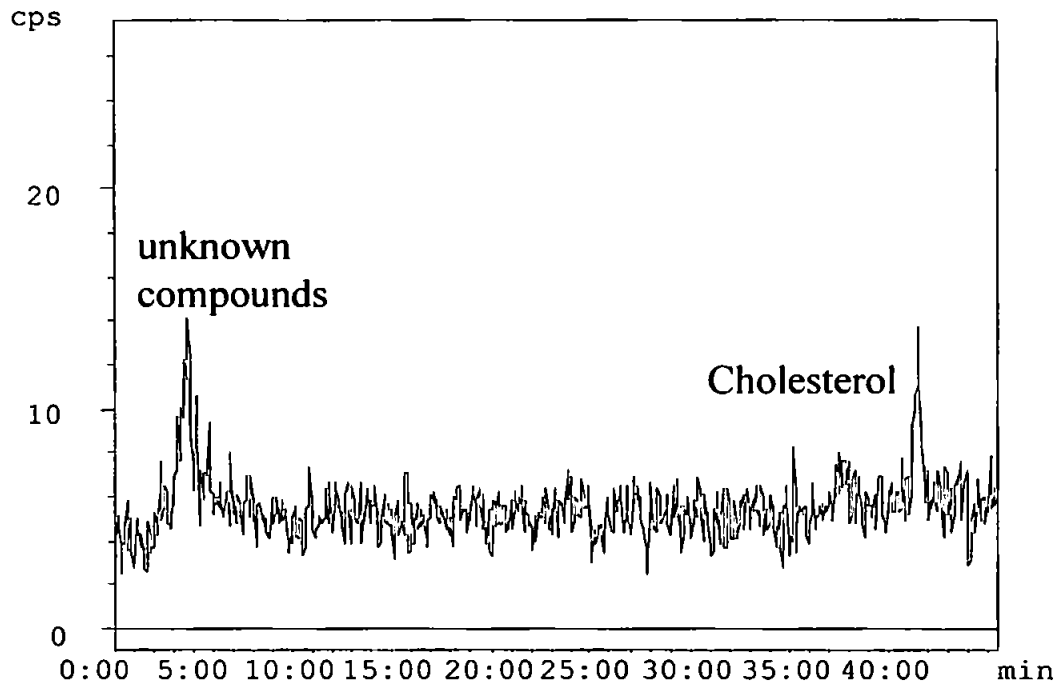


Figure 4.16 rHPLC of SPE extracted liquid sample, 24 h DA incubation, showing unknown early eluting compounds (3.4 minutes) and 26-^{14}C cholesterol (**III**) (40.2 minutes).

Two fractions, 0-5 and 38-42 minutes were taken from all SPE extracts for rGC analysis, as TMS derivatives but no radioactive components were detected above the LOD.

SPM samples, which contained the majority of radioactivity, were extracted with methanol (x 5) and each extract fractionated by rHPLC (Figure 4.17).

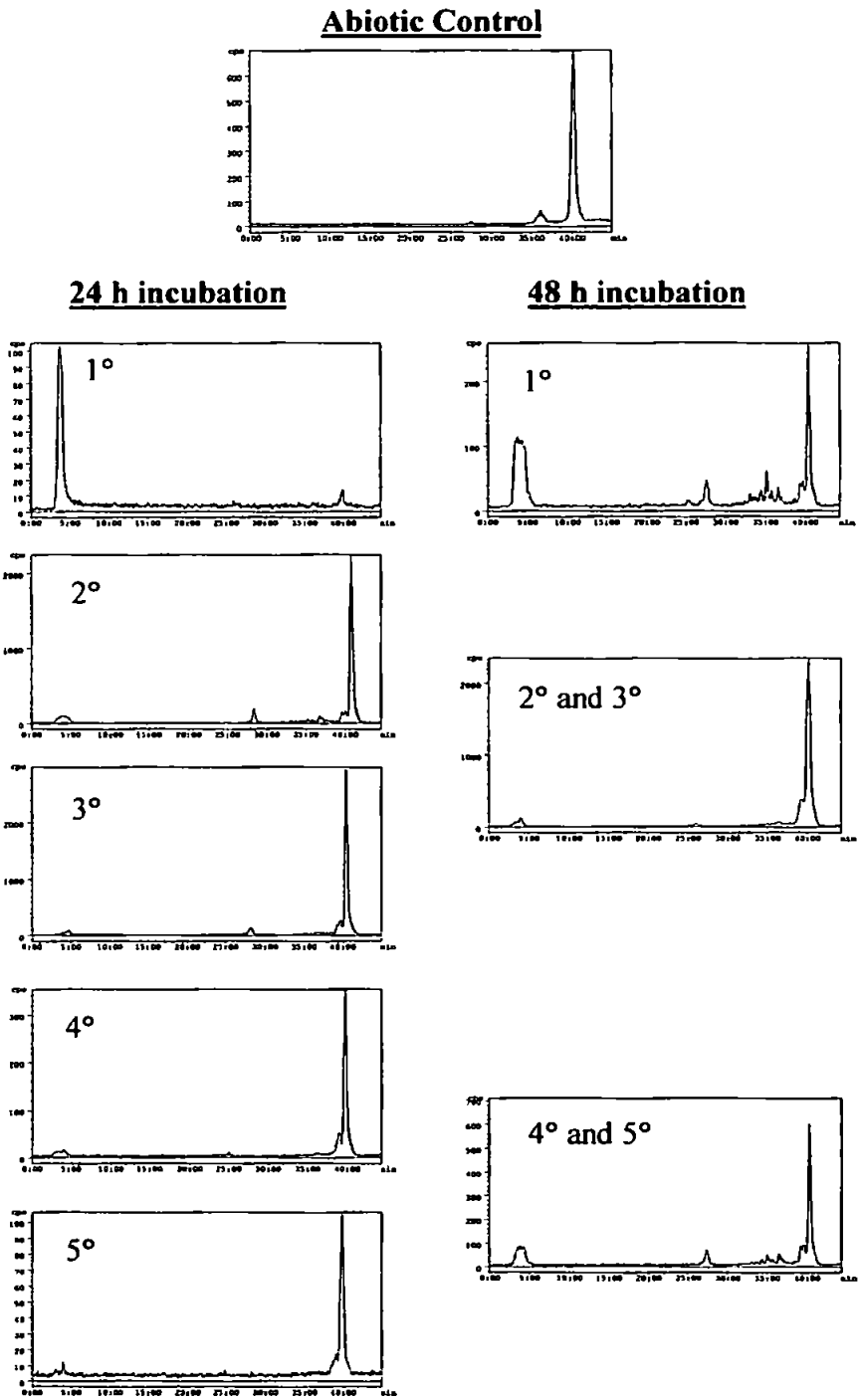


Figure 4.17 rHPLC analysis of the DA aerobic incubations, first to fifth solvent extracts of SPM, 24 h (20 % of total injected activity) and 48 h (21 % of total injected activity) compared with an abiotic control (22 % of total injected activity).

The primary extracts had a high water content and contained mainly early eluting polar compounds. Just 1.5 % of total activity in the SPM was associated with the first extract (24 h) compared with 14 % for the 48 h first extract. The secondary and tertiary extracts showed the majority of total activity added whereas no further activity was obtained after the fifth extract (Table 4.2). The abiotic control showed that there was a small amount of abiotic products, *ca* 10 % excluding mineralisation to CO_2, which accounted for about 2 % of the total activity. However, due to the low activity of the abiotic product identification *via* rGC was not possible.

For the 24 and 48 h DA incubations five further fractions were taken from each rHPLC as shown in Figure 4.18. Fractions from the five 24 h extracts and the three 48 h extracts were pooled for rGC analysis (as the TMS ether derivatives).

Extract	**24 h incubation (% of total activity)**	**48 h incubation (% of total activity)**
1st	1.5	14
2nd	41	63
3rd	50	
4th	5.8	23
5th	1.5	

Table 4.2 The activity associated with each extraction of SPM from the 24 h and 48 h DA incubations.

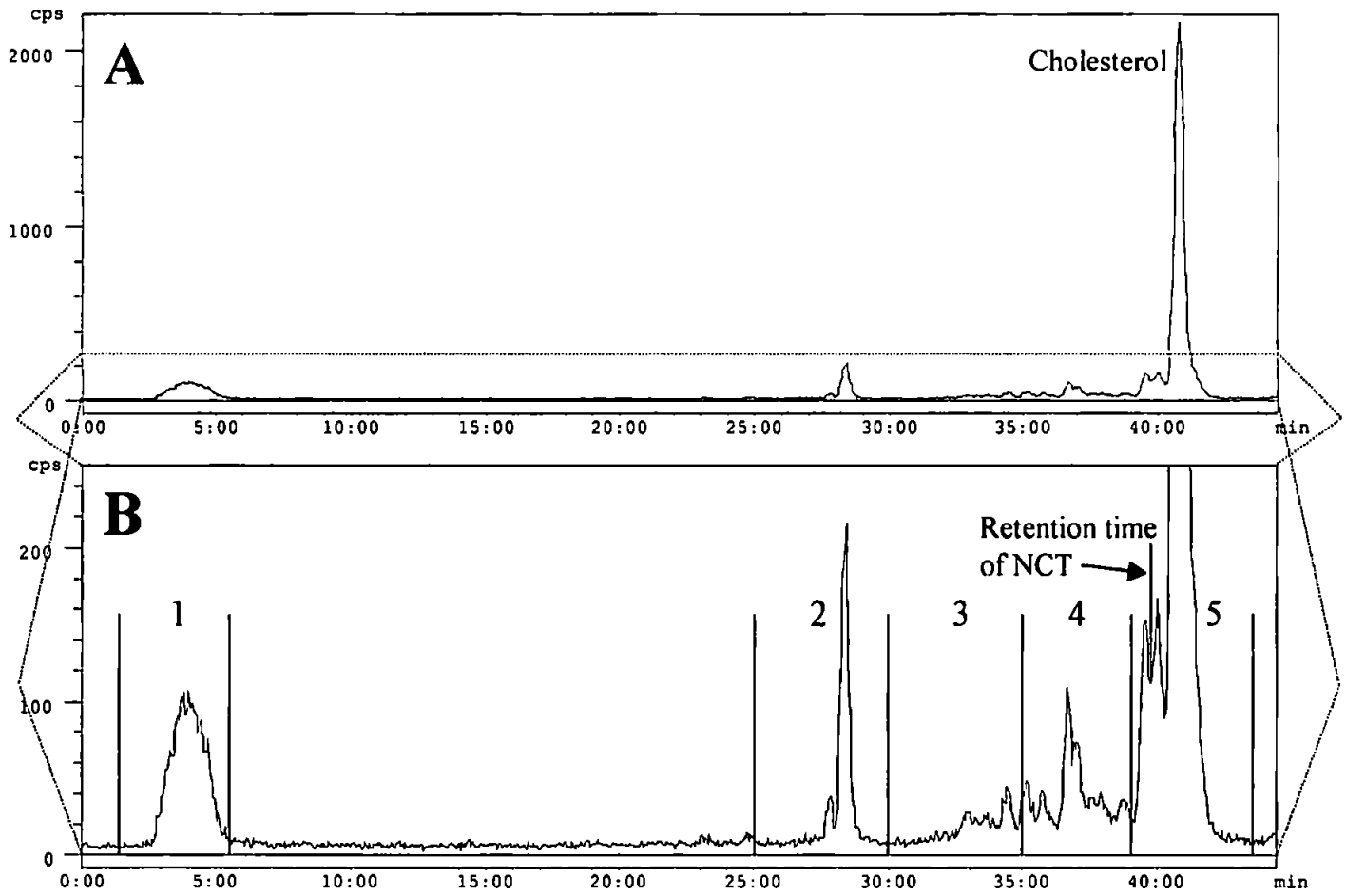


Figure 4.18 rHPLC fractionation of 24 h DA solid extract. Chromatogram B is a magnification of chromatogram A. B shows the 5 fractions taken for rGC analysis. The retention time of NCT is also highlighted.

4.3.2.1 Fraction 1

Fraction 1 of the 24 h and 48 h DA solids sample eluted between 0-5.5 min. contained early eluting compounds which were unresolved by HPLC (Figure 4.18).

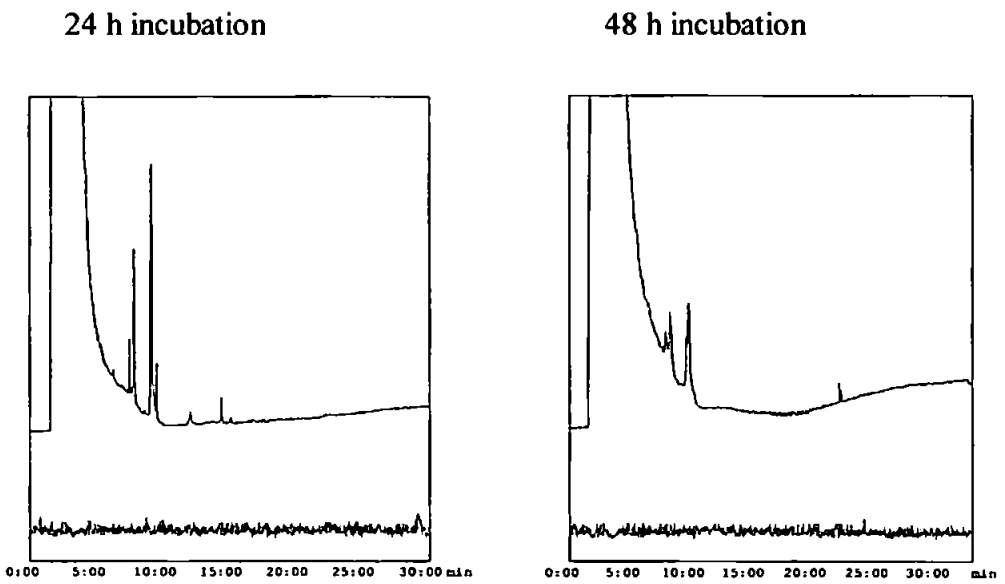


Figure 4.19 GC-FID and rGC chromatograms of rHPLC fraction 1 (0 – 5.5 min), 24 and 48 h aerobic incubations, showing a series of peaks between 7 and15 minutes detected only by FID (black line). The activity on the corresponding RD chromatogram (red line) was <LOD.

rGC analysis of fraction 1 from 24 h and 48 h incubations revealed no radioactive components (Figure 4.19). It is likely that these compounds which from their HPLC elution behaviour are polar components, are not amenable to GC analysis.

4.3.2.2 Fraction 2

rHPLC fraction 2, of the 24 h and 48 h DA solids samples eluted between 25-30 minutes and contained two radioactive components in the second to fourth extracts (Figure 4.17 and 4.18). When collectively analysed by rGC three radioactive

metabolites were seen, corresponding to three components observed by FID (Figure 4.20).

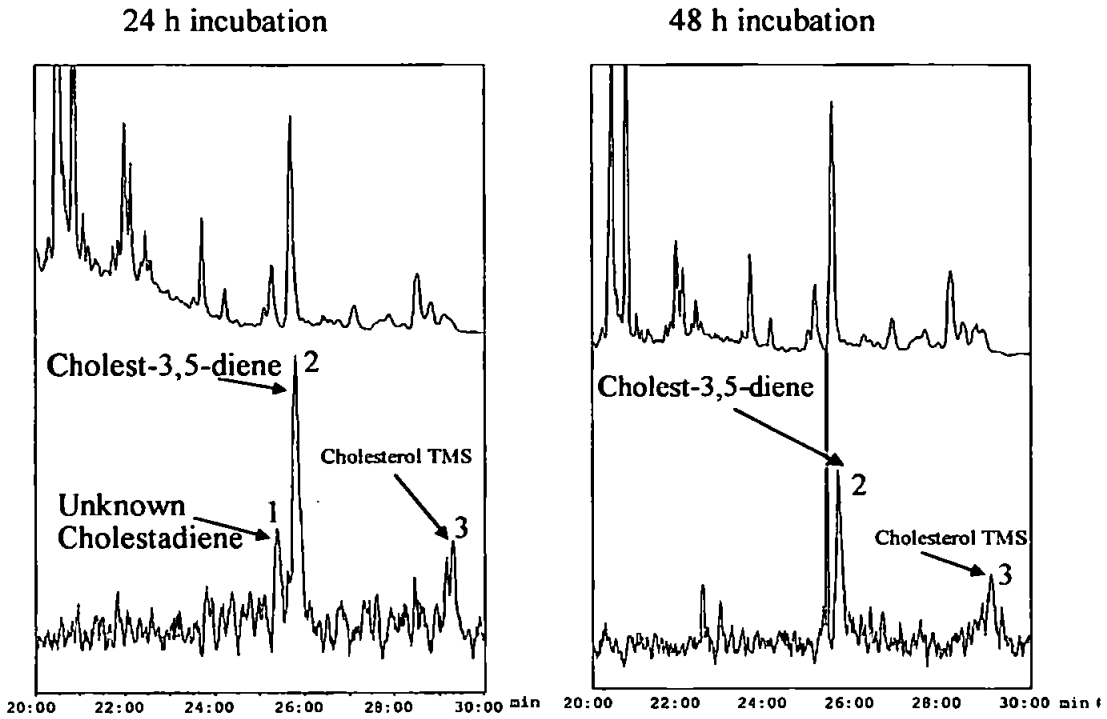


Figure 4.20 GC-FID and rGC analysis of DA aerobic incubations, 24 and 48 h, rHPLC fraction 2 (25 – 30 min), as the TMS ether derivatives.

The retention indices of the three radioactive metabolites observed by rGC on DB-5 stationary phase are given in Table 4.3.

Peak	Retention Index (GC-FID) DB-5 1 μm	Retention Index (GC-MS) DB-5 0.25 μm
1	2924	2909
2	2957	2940
3	3198	3194
Cholesterol TMS	3205	3193
Coprostanol TMS	3115	3089
Cholest-3,5-diene	not determined	2942

Table 4.3 Retention indices of radioactive components and corresponding components identified by FID and GC-MS on the same colum phases compared with pure reference analytes as the TMS ether derivatives.

The mass spectra of components 1 and 2 both had molecular ions of *m/z* 368 (Figure 4.21). No alteration in $M^{+\cdot}$ was observed when samples were analysed without BSTFA derivatisation suggesting the absence of derivatisable groups. The mass spectrum of component 2 also contained a large fragment ion *m/z* 147 attributed to cleavage of the C-ring leaving an unsaturated A,B-ring ion (Figure 4.21 and 4.22). The retention index was close to that of cholest-3,5-diene (**LXXXIX**) and the compounds co-eluted on co-injection. Thus component 2 was assigned to cholest-3,5-diene (**LXXXIX**), by co-injection with commercially available **LXXXIX**, component 1 to an unspecified isomer and peak 3 when examined by GC-MS had a $M^{+\cdot}$ ion of *m/z* 458 and base ion was *m/z* 368 (Figure 4.21). The retention indices of 3194 and 3206 were in good agreement to those found herein (Table 4.3) for cholesterol (**III**). The mass spectrum is consistent with this.

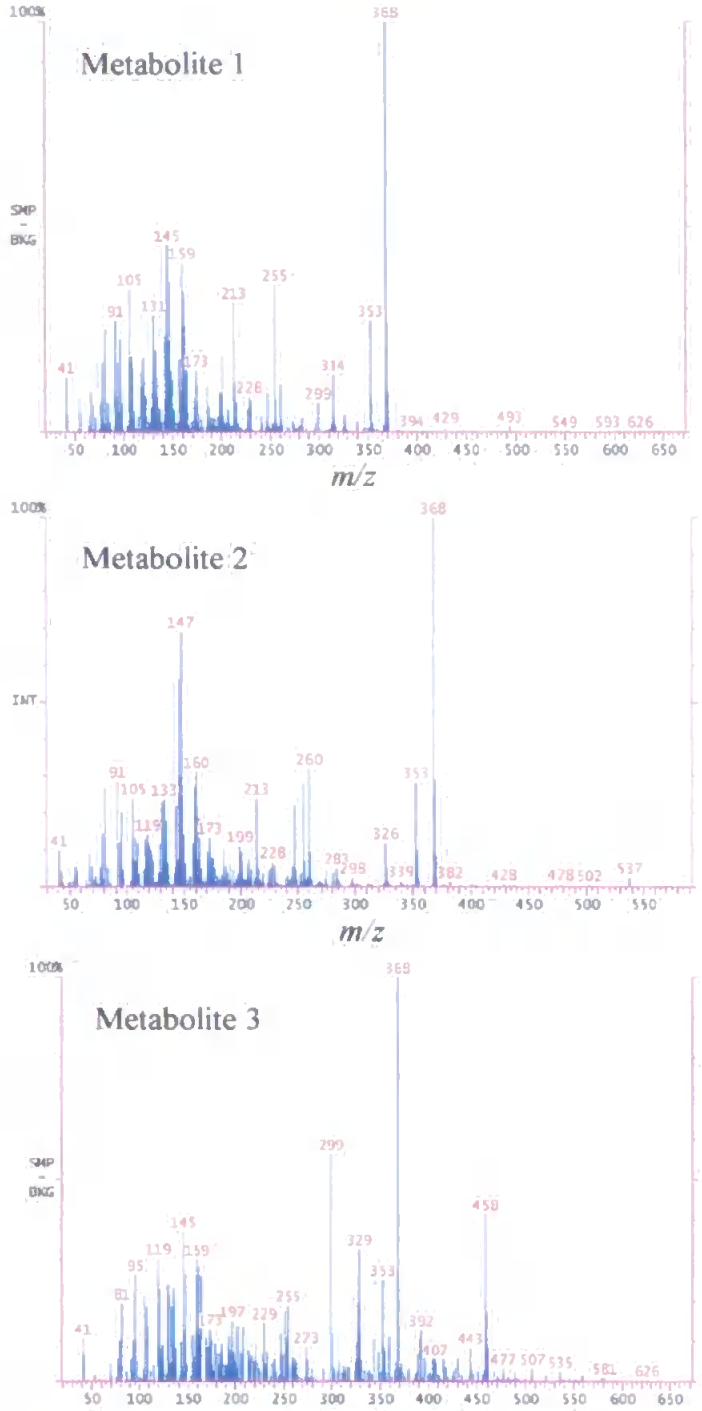


Figure 4.21 Mass spectra of the three metabolites detected in SPM extracts, rHPLC fraction 2, from 24 and 48 h DA incubations with 26-^{14}C-cholesterol (**III**). Metabolite 1 was an undetermined compound, metabolite 2 was identified as cholest-3,5-diene and metabolite 3 as **III**.

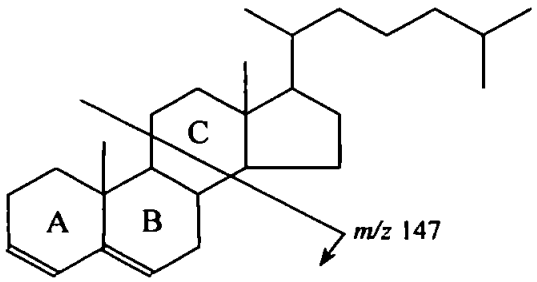


Figure 4.22 C-ring cleavage of cholest-3,5-diene leaving an A/B-ring fragment ion.

4.3.2.3 Fractions 3 and 4

Fraction 3, 30-35 minutes, contained three significant rHPLC components. However, the compounds were of low activity, ranging from 30 to 45 cps. Fraction 3 was analysed by rGC but even with the detector-split ratio set to 100 % RD the activity was <LOD.

Fraction 4, 35-39 minutes, generally contained above background activity but four distinct metabolites with retention times and specific activities of 35:03 (46cps), 35:39 (40 cps), 36:36 (108 cps) and 36:56 (73 cps) (Figure 4.18) were seen. When the fraction was analysed by rGC, two peaks were observed (Figure 4.23).

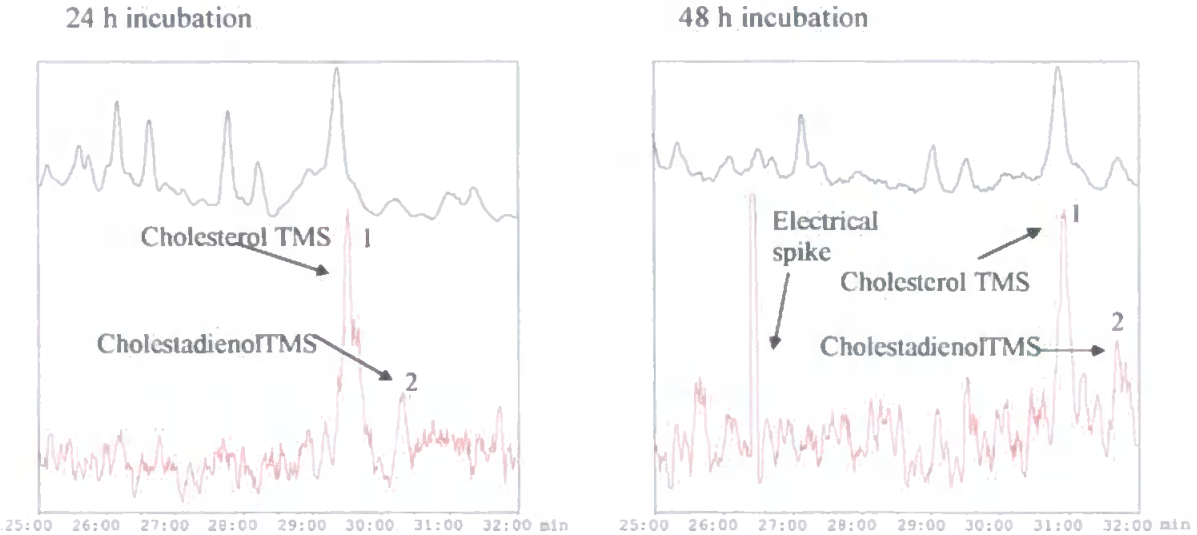


Figure 4.23 GC-FID and rGC analysis of rHPLC fraction 4 as the TMS derivative. Cholesterol (**III**) and a undetermined cholestadienol are shown.

Metabolite 1 coincided with 26-^{14}C cholesterol (**III**). The smaller component 2, had a retention index of 3236. When the same fraction was examined by GC-MS a compound with a molecular ion of *m/z* 456, retention index of 3234, was observed (Figure 4.24).

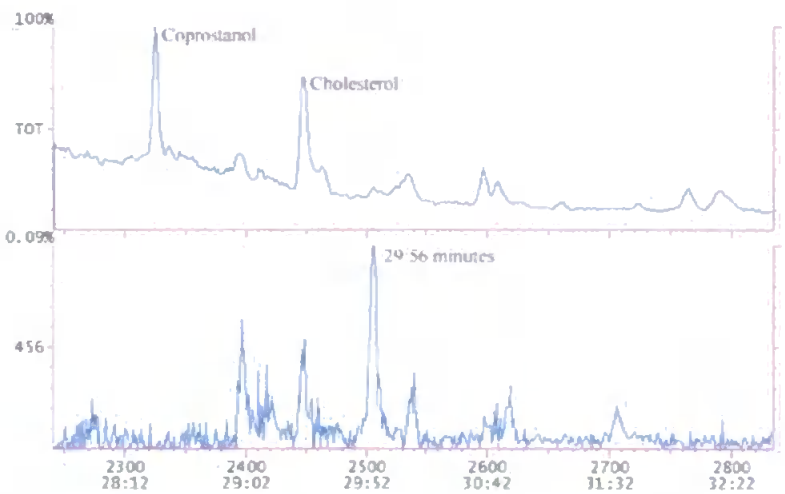


Figure 4.24 GC-MS analysis of fraction 4 shows the appearance of cholesterol (**III**) and a compound with a M^+ of *m/z* 456 (as TMS ether derivative).

The mass spectrum of compound eluting at 29:56 minutes is shown in Figure 4.25.

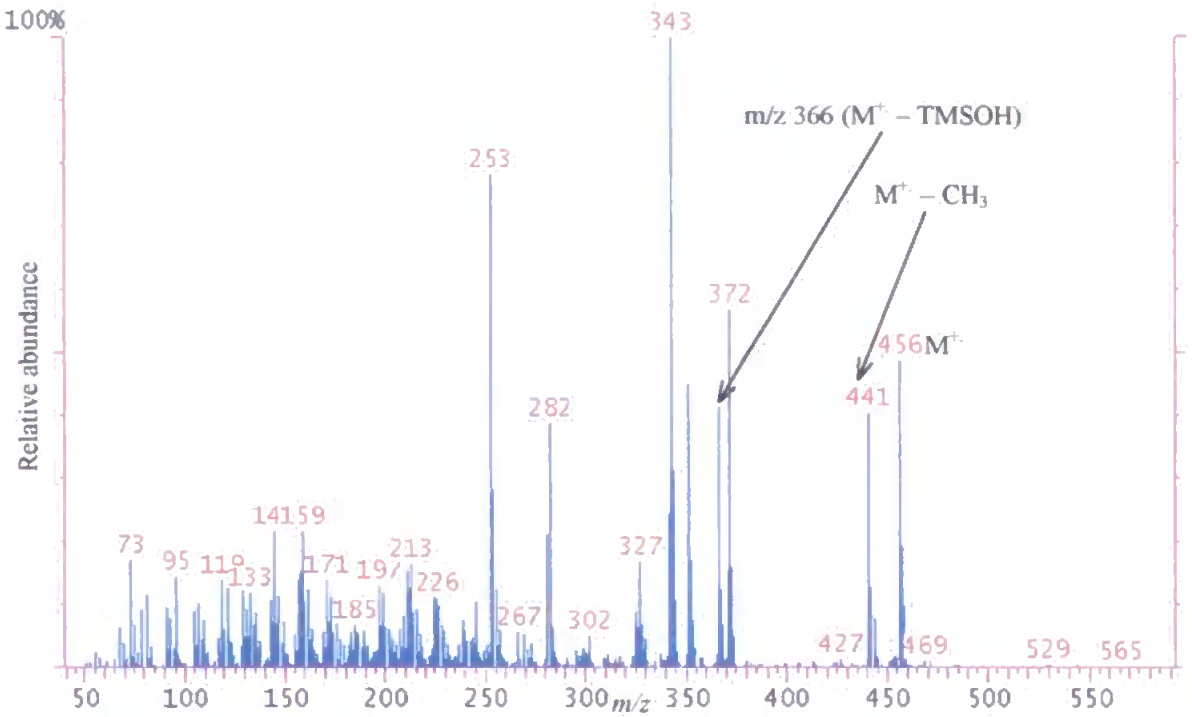


Figure 4.25 Mass spectrum of unknown compound eluting at 29:56 minutes (Figure 4.24) , rHPLC fraction 4 (35 – 39 min).

The molecular weight of cholesterol (**III**), as the TMS ether derivative, is 458. Thus the unknown compound could be a diene of **III** such as cholest-5,7-dien-3β-ol (**LXXXX**). Harvath and Kramli (1947) showed **LXXXX** to be a metabolite of **III** when incubated with *Azotobacter*. However, when the TMS ether derivative of **LXXXX** was examined by GC-MS the retention index was 3229 ± 0.6 compared with 3234 for the unknown metabolite and the mass spectrum did not match that of unknown compound 2 (Figure 4.26).

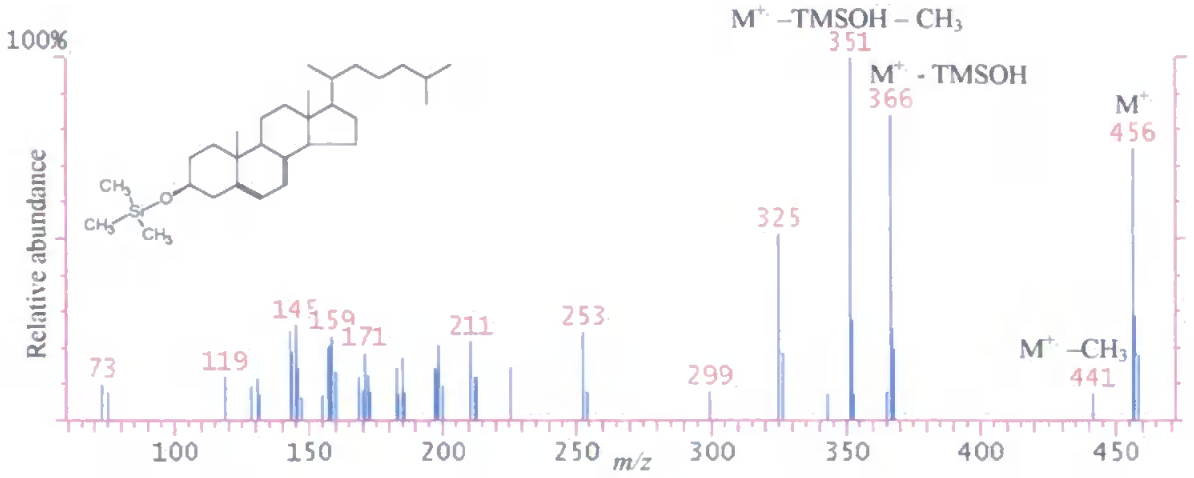


Figure 4.26 Library mass spectrum of cholest-5,7-dien-3β-ol TMS (**LXXXX**).

No further cholestadienols were commercially available and hence the compound eluting at 29:56 minutes (Figure 4.24) was not fully identified but tentatively identified as a cholestadienol isomer other than the 5,7-isomer.

4.3.2.4 Fraction 5

Fraction 5, 39-44 min, contained most of the major sterols such as cholesterol (**III**), coprostanol (**LXXII**) and β-sitosterol (**XXXI**), and was the most radioactive fraction (~75 % of all activity determined between 39-44 min) (Figure 4.18). NCT (**LXXXI**), if present, would also be expected to be in this fraction. Figure 4.27 shows the rGC chromatogram of fraction 5. The same fraction was analysed by GC-MS (Figure 4.28).

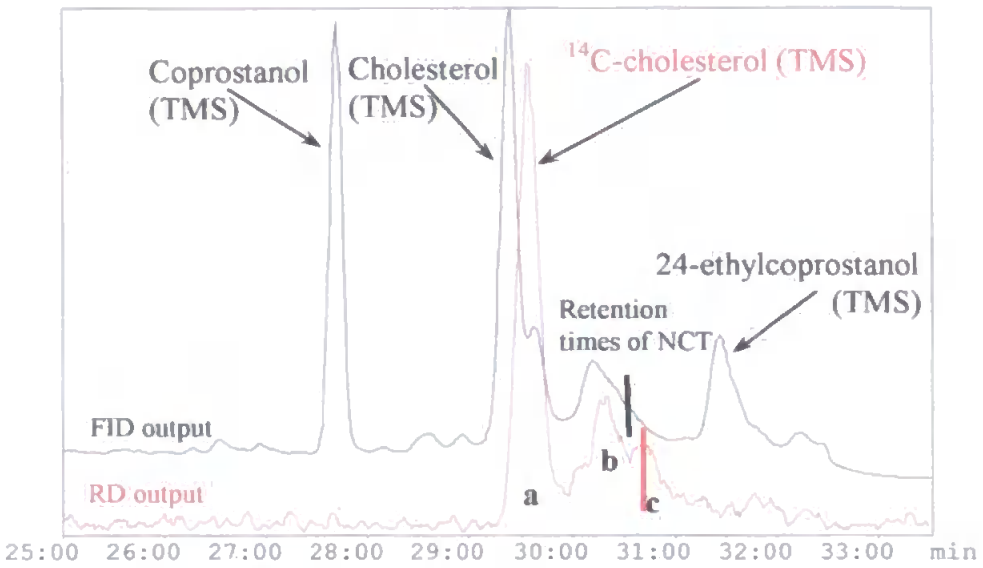


Figure 4.27 Partial GC (FID and RD outputs) for rHPLC fraction 5 (39 – 44 min), 24 h DA incubation of 26-^{14}C-cholesterol.

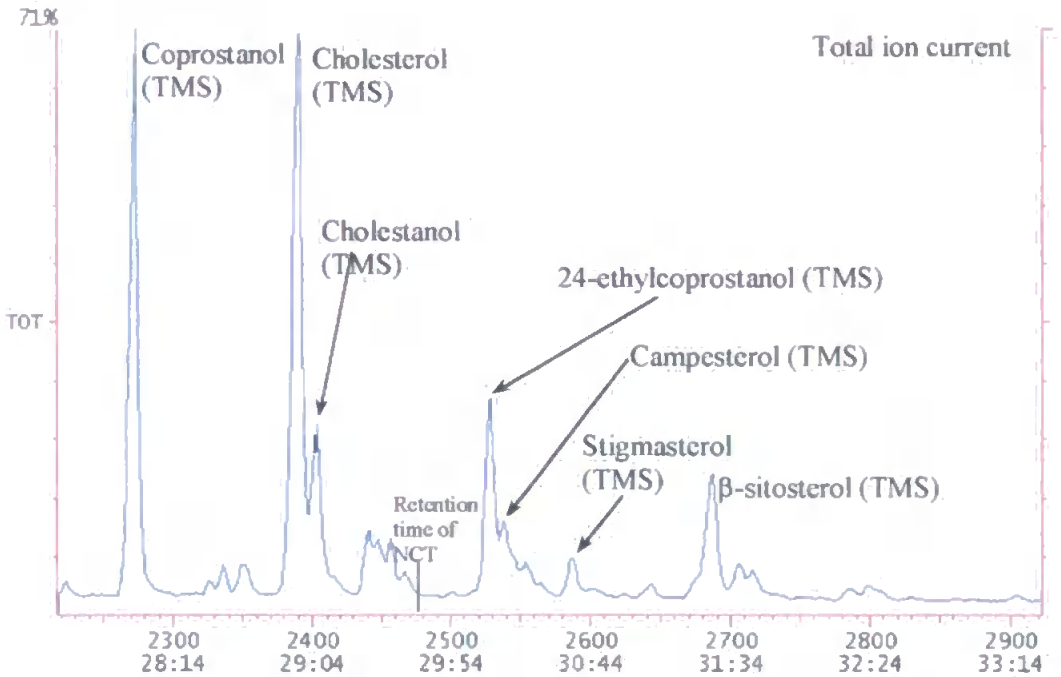


Figure 4.28 Partial GC-MS total ion current chromatogram rHPLC fraction 5 (39 – 44 min). The retention time of NCT (**LXXXI**) is shown.

Figure 4.27 shows that three radioactive metabolites (a – c) were present. The major radioactive component within the fraction was cholesterol (**III**) (peak a). However,

two metabolites were observed to elute between 30-31 minutes (peaks b, c). Comparing rGC, FID and GC-MS TIC chromatograms of this fraction it was apparent that rGC radioactive peak b (RT 30:2 minutes) corresponds to the first peak observed by GC-MS in the radioactive region (Figure 4.29). This component had a mass spectrum with an apparent $M^{+\cdot}$ *m/z* 456 (RT 29:25 minutes, GC-MS) and was assigned to a cholestadienol. Radioactive peak c (Figures 4.27, 4.29) correlates with a component with an apparent $M^{+\cdot}$ *m/z* 458 (RT 29:37 minutes, GC-MS) in the GC-MS TIC chromatogram and was tentatively assigned to a cholestenol (other than cholesterol). Although this component also co-eluted with NCT (**LXXXI**) (Figure 4.27), no mass spectral evidence could be found to support the identification as **LXXXI**. It is entirely possible that the amount of **LXXXI** produced (if any) was below the limits of detection of GC-MS however. Thus the proof of the metabolic route from cholesterol (**III**) to **LXXXI** remains tentative.

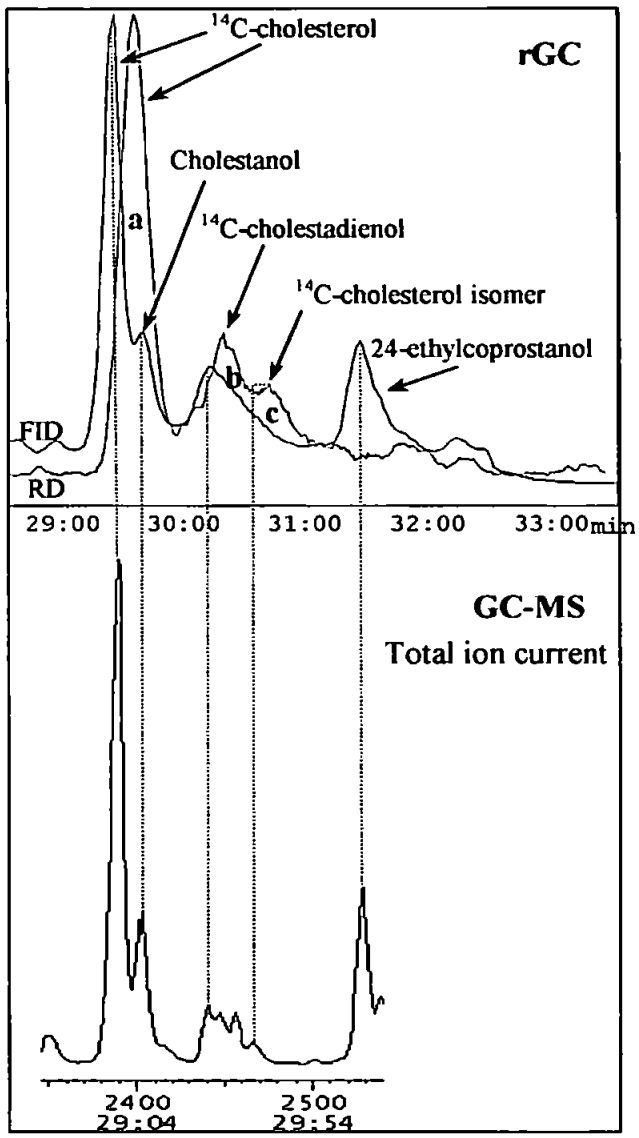


Figure 4.29 Combined partial rGC and GC-MS, total ion current, chromatograms from rHPLC fraction 5 (39 – 44 min) of 24 h DA incubation with 26-^{14}C-cholesterol (**III**). Common peaks of interest are highlighted (a, b and c).

In an attempt to further remove unwanted, non-radiolabelled compounds from this HPLC fraction 5 (*i.e.* to increase radioactivity to the rGC), a rTLC step was introduced on a freshly extracted 24 h DA solid sample. The sample was fractionated by rHPLC (as before) and two fractions were taken at 35-40 minutes (**A**) and 40-44

minutes (**B**) in a bid to separate cholesterol (**III**) metabolites, possibly including NCT (**LXXXI**) from unaltered **III** and steroids of similar retention times (Figure 4.30).

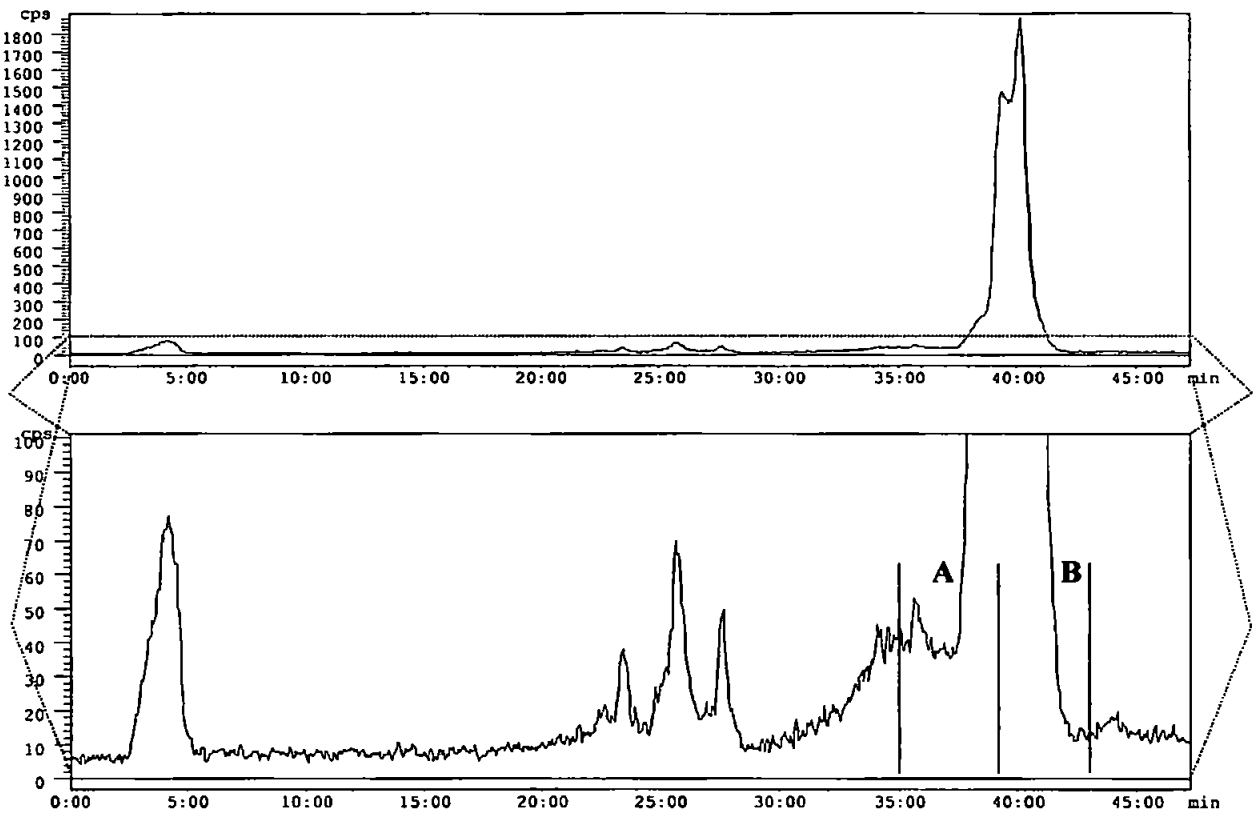


Figure 4.30 rHPLC fractionation of a 24 h DA solid extract. Fractions A (35-40 min) and B (40-44 min) were collected for rTLC analysis.

Fractions A and B were re-chromatographed by rTLC over silica with DCM as mobile phase (Figure 4.31).

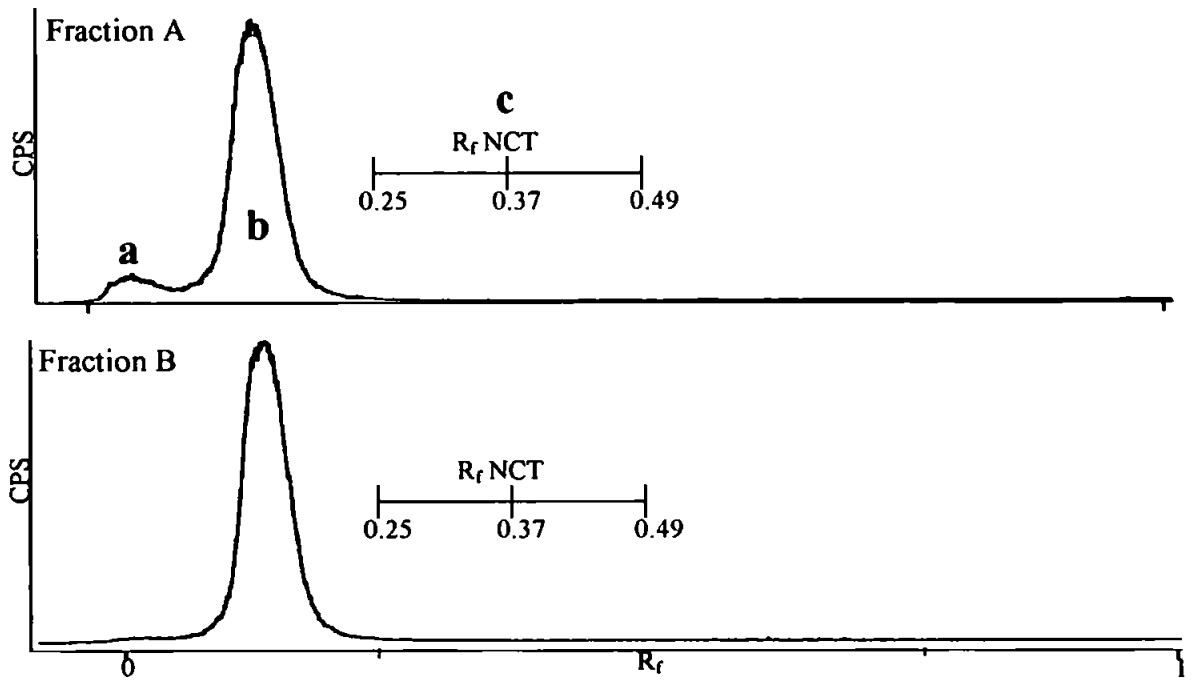


Figure 4.31 rTLC of rHPLC fractions A and B (Figure 4.30) from solid extracts of 24 h DA incubation with 26-^{14}C-cholesterol. Three main regions or 'peaks' **a**, **b** and **c** of interest are highlighted. Region **c** indicates the broad R_f for NCT under the conditions used.

The R_f value for NCT (**LXXXI**), under the rTLC conditions, was determined to be 0.37 ± 0.12 (Figure 4.31; region **c**). However, no radioactive peaks were identified in this region in either fraction A or B. The NCT region, (R_f 0.25-0.49) from fractions A and B were nonetheless extracted with methanol, derivatised with BSTFA and examined by rGC. Not surprisingly no radioactive NCT was detected. The only metabolites obtained in region **c** fraction B when analysed by rGC was traces of unmetabolised cholesterol (**III**). The two rTLC radioactive components in fraction A ('peaks' **a** and **b** ; Figure 4.31) were analysed by rGC. The first, minor component **a** was not detectable. However, the major rTLC 'peak' b resulted in three distinct rGC compounds (Figure 4.32).

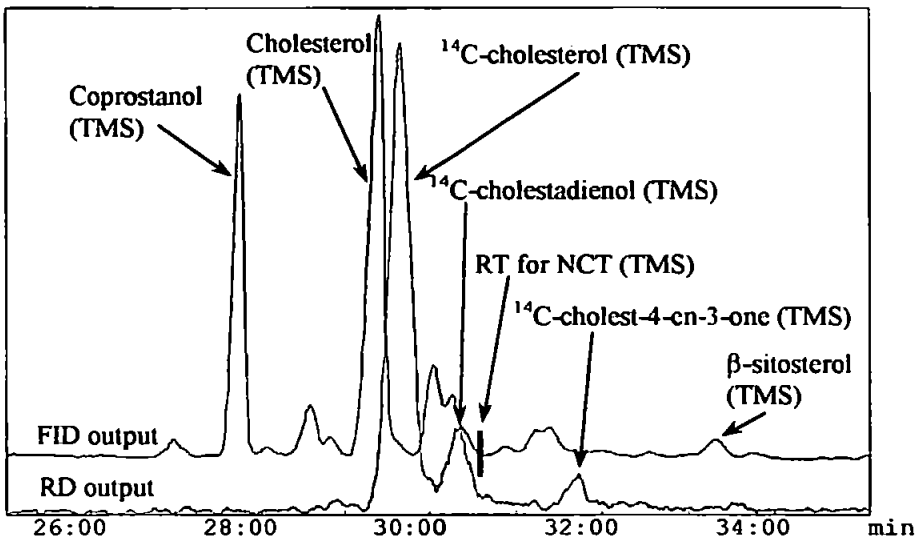


Figure 4.32 Partial rGC chromatogram of rTLC fraction A 'peak' **b** as the TMS derivative. The RT of NCT TMS is indicated on the FID output.

The component in fraction B, when analysed by rGC as the TMS ether derivative, showed that the entire activity was due to unmetabolised 26-^{14}C **III**. The fraction was also analysed by GC-MS and the largest peak was identified as cholesterol (**III**) (Figure 4.33). The second radioactive metabolite, retention time 30:18 minutes, again assigned as an undetermined dienol ($M^{+\cdot}$ *m.z* 456 as the TMS ether derivative *cf.* Figure 4.29) with a retention time of 30:01 minutes. The third radioactive metabolite at retention time 31:41 minutes was either due to another undetermined dienol (retention time 31:13 minutes; GC-MS) or cholest-4-en-3-one (**LVII**) (retention time 31:21 minutes; GC-MS). On the basis of retention time and lag time between the detectors it is reasonable to assume the third component was cholest-4-en-3-one. Thus no evidence was found for NCT production from **III** in this 48 h study

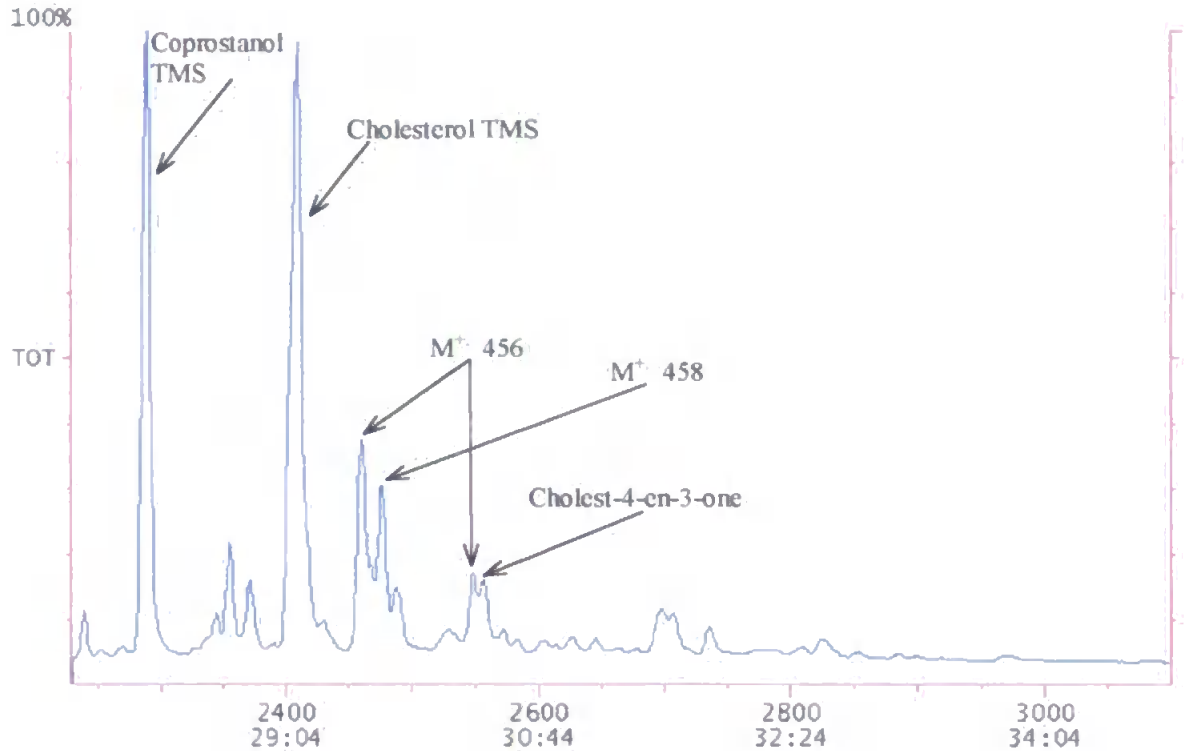


Figure 4.33 Partial GC-MS, total ion current, chromatogram of rTLC fraction A 'peak' **b** (Figure 4.31), 24 h DA incubation with 26-^{14}C-**III** solid extract, (as the TMS ether derivative).

4.4 Discussion

As reviewed in Chapter 1 there have been numerous studies of the metabolism of cholesterol (**III**) as a sole carbon source using single microorganisms (Harvath and Kramli, 1947, 1948; Wang and Sih, 1963; Chang and Sih, 1964; Sih *et al.*, 1965a,b,c; Sih, *et al.*, 1967a,b; Sih *et al.*, 1968; Nagasawa *et al.*, 1969; Arima *et al.*, 1978; Martin, 1977; Bhattacharyya *et al.*, 1984; Madyastha and Shankar, 1994). However, the examination of metabolites of sterols, including **III**, during sewage treatment containing a suite of microorganisms is much more limited (Gaskell and Eglinton, 1975; McCalley *et al.*, 1981; Taylor *et al.*, 1981). The use of equipment such as the

SCAS system is a useful tool to initially examine biodegradation of a test compound in question. The percentage removal rates do not, however, fully correlate with continuous-feed activated sludge (CAS) system and poorly mimic a fully operational STW due to aeration rates and sludge retention times (Boethling *et al.*, 1997). Furthermore, the SCAS system does not take into account biodegradation occurring in receiving waters prior to entering a STW. The findings of Boethling and co-authors were based on the metabolism of synthetic chemicals for the means of risk assessing toxicity and did not mention the possible uses for natural compound biodegradation. For cholesterol (**III**), entering a STW at high concentrations has been shown, herein, to be rapidly biodegraded. For the current study the use of the SCAS system enabled a thorough examination of **III** metabolism furthering our understanding and was regarded as both a cost effective and useful first examination. The anaerobic metabolism of **III** was performed on a much simpler scale with 4-^{14}C **III** being added to sealed vessels containing anaerobic activated sludge in an anoxic medium. The vessels were simply incubated at ~ 30 °C over a course of 9-weeks.

Solid and liquid samples of both the aerobic and anaerobic experiments were extracted by standard techniques. The solid samples were solvent extracted with a high degree of efficiency, whereas the liquid samples, containing <5 % of the total activity, had only a limited efficiency when using SPE (~ 50 % of total activity in solution was retained by SPE). The products that remained in solution were not identified. The extracts were initially fractionated by rHPLC and subsequent fractions of interest were collected and analysed by rGC followed by full scan GC-MS. The rHPLC and rGC were calibrated using stock ^{14}C cholesterol (**III**) solutions of specific activity. The rHPLC was shown to have a high degree of internal quenching (~80 % activity quenched when examining

peak height:specific activity ratio). The low efficiency of the rHPLC was, however, generally over-come by the elevated sample volume that could be used (up to 2000 μL). Furthermore, the rHPLC had an insignificant dependence on the amounts of natural sterols in the extract as the column did not appear to be saturated with sample material. The rGC had an efficiency of 45-85 % (based on specific activity: peak height ratio) depending on the split ratio between FID and RD employed. The rGC was highly sensitive to other natural products in the sample and thus there was a physical limitation to the amount of sample that could be analysed, this was shown particularly with anaerobic extracts. The rGC was, however, sensitive enough to be able to determine a number of radioactive metabolites of aerobic metabolism. Furthermore, direct comparisons and interpretations between the rGC and GC-MS could be made as the same column phase, DB-5, was used throughout. Thus coupled with the determination of a selection of retention indices on each instrument and used in conjunction with the comparable chromatograms it was possible to identify metabolites.

4.4.1 Anaerobic metabolism

The anaerobic metabolism of cholesterol (**III**) was examined over the course of 9 weeks with triplicate samples taken weekly for analysis. The anaerobic sludge contained most activity and if NCT (**LXXXI**), with a computed log K_{ow} of >9, was indeed a metabolite of **III** it was expected to be bound to SPM. The SPM samples were extracted with methanol and initially fractionated by rHPLC. For all samples analysed two radioactive metabolites were observed in close proximity to each other (Figure 4.2). The fraction taken, containing both metabolites, was analysed by rGC. The results were inconclusive due to the high abundance of natural steroids masking

the radiolabelled portion (Figure 4.3). For the anaerobic study it was apparent that an insufficient amount of radiolabelled **III** was initially added to each vial (4.9 KBq $vial^{-1}$) when compared with the significant natural steroid content. In an attempt to overcome this problem a greater volume of sample was analysed. However, this caused severe contamination problems. Therefore, for significant radioactivity to be >LOD for the rGC a high proportion of the natural steroid burden had to be removed. The inclusion of rTLC, therefore, allowed partial removal of interfering compounds and in itself gave tentative evidence for the possible biosynthesis of NCT (**LXXXI**) (Figure 4.4 and 4.5) over two separate solvent systems (coprostan-3-one (**LXXXIII**) and **LXXXI** had similar R_f values over the two solvent systems). However, when the rTLC fractions were analysed by rGC the presence of NCT (**LXXXI**) was not proven (Figure 4.6). There was, however, a large coprostan-3-one (**LXXXIII**) peak seen by FID (Figure 4.6). It is subjective whether **LXXXIII** was the major radiolabelled metabolite shown by rTLC (Figure 4.5). The fractions were analysed by GC-MS and again **LXXXI**, as the TMS ether, ($M^{+\cdot}$ 440), was not determined (Figure 4.7). Cholesterol (**III**) was itself only a minor constituent of the anaerobically metabolised sludge extracts (Figure 4.7).

The concentration of available 4-^{14}C-cholesterol (**III**) was low, however, the overall cholesterol (**III**) burden in the sludge prior to anaerobic incubation was considerable. Thus if NCT (**LXXXI**) was a metabolite of anaerobic **III** biodegradation it might be implied that the amount of **LXXXI** determined was dependent on the total **III** content(^{12}C + ^{14}C). Thus the ratio of 4-^{14}C **LXXXI** and 4-^{12}C **LXXXI** should mimic the 4-^{14}C:4-^{12}C **III** ratio in the sludge prior to incubation (assuming **LXXXI** bioaccumulates). The amount of 4-^{14}C **III** originally added was equivalent to 10 μg

sample $^{-1}$ compared with *ca* 1 mg 4-^{12}C **III** present in the sludge of each sample. Therefore, under ideal conditions the 4-^{14}C:4-^{12}C **LXXXI** ratio would be 1:100. Thus, GC-MS analysis of the sludge extract post incubation would probably be more sensitive in determining 4-^{12}C **LXXXI** than rGC in determining 4-^{14}C **LXXXI.** NCT (**LXXXI**) was not identified as a metabolite, by GC-MS in all extracts analysed, as a result of **III** biodegradation. If the original hypothesis was correct and **LXXXI** was an anaerobic **III** metabolite the only rational explanation was either **LXXXI** was a reactive intermediate that does not bioaccumulate or was an extremely minor product (*i.e.* <LOD for GC-MS).

The examination of >60 % of the anaerobic samples failed to prove the existence of NCT (**LXXXI**) or identify any cholesterol (**III**) metabolites. Comparing the results reported herein to those of Gaskell and Eglinton (1975), who incubated ^{14}C-cholesterol in anaerobic sludge, and Taylor *et al.* (1981), who used culture experiments (with **III** as sole carbon source) and added anaerobic sediment slurry with ^{14}C III, it was apparent that the limiting factor was the concentration of ^{14}C-cholesterol (**III**) used in the present study, 4.9 KBq, compared with 126 KBq and 37 KBq averaged values used by Gaskell and Eglinton, and Taylor and colleagues respectively. With total concentrations of ^{14}C **III** far greater than those used herein (26 and 8 fold decrease in activity) the authors were able to identify coprostanol (**LXXII**) (5α and 5β H) and cholest-4-en-3-one (**LVII**) as major metabolites. Taylor *et al.* (1981) was also able to identify androst-4-en-3,17-dione (**XXXXIX**), 5α-androstan-3,17-dione (**LXXXXI**) and identified that 2-5 % of the total 4-^{14}C **III** added was recoverable as $^{14}CO_2$. The mineralisation of 4-^{14}C **III** herein was not examined. The average extraction of the

total anaerobic samples was *ca* 82 % (Figure 4.1) and over the course of the incubation period there was a steady decrease in available activity from the SPM. The shortfall could be due to $^{14}CO_2$ or $^{14}CH_4$ production dissolved in the liquid or in the headspace. The addition of radiolabelled cholesterol herein was low, however, the addition of excess radiolabelled substrate could perturb the system increasing the overall mass present.

4.4.2 Aerobic metabolism

The study of the aerobic metabolism of cholesterol (**III**) required the use of a bench scale activated sludge treatment system. Two systems were employed, the semi-continuous activated sludge unit (SCAS) and the Die Away unit (DA). The SCAS unit works similar to a true treatment plant allowing the microorganisms to proliferate by the addition of fresh influent and synthetic feed once every 23 h (rather than continuous as with a true STW). The DA unit uses fresh influent, solely, without the addition of feed and hence the life expectancy of the microorganisms is greatly reduced (the SCAS system can be kept running optimally indefinitely). In the present study the SCAS unit was only run for 96 h, with sub-samples taken every 24 h, due to the rapid dilution rates of original 4-^{14}C-cholesterol (**III**) added (50 % of liquid and 10 % of solids removed for analysis daily) and the rate of mineralisation. The DA was not used for the same time period due to the reduction in microorganism performance and hence was only run, optimally, for 48 h (Dr. J. Snape, *personal communication*). The positioning of ^{14}C radiolabelled in the cholesterol (**III**) molecule differed between the two experiments. The SCAS unit used 4-^{14}C **III** (same as anaerobic study) while the DA system used 26-^{14}C **III**. The two radiolabelled cholesterols were used in an attempt to identify products of both side-chain cleavage and A-ring rupture.

The percentage mineralisation of 4-^{14}C-cholesterol (**III**) in the SCAS and 26-^{14}C-**III** in the DA over the first 24 h of incubation was 29 % and 21 % respectively (Figures 4.8 and 4.13). The CO_2 traps contained no ^{14}C volatile species. Therefore, there is evidence for both alkyl side chain cleavage and A-ring opening releasing the ^{14}C atom as carbon dioxide. The microorganism induced oxidative cleavage of the alkyl side chain has been examined by a number of authors (Sih *et al.*, 1967a,b; Sih *et al.*, 1968; Nagasawa *et al.*, 1969; Martin 1977; Bhattacharyya *et al.*, 1984) using a single microorganism and sole carbon source. The route of side chain cleavage is well understood. The mechanism initially proceeds *via* the oxidative cleavage of the C24-C25 bond, producing a propanoic acid (**LXI**) molecule (from C25, C26 and C27), followed by the subsequent C22-C23 and C17-C20 bond cleavage resulting in the formation of a molecule of ethanoic acid and **LXI** respectively. The first **LXI** molecule formed contains the radiolabelled atom used in the current study and hence must be further oxidised to yield ^{14}C carbon dioxide (Sih *et al.*, 1967a,b; 1968). The stepwise cleavage of the alkyl chain outlined above results in the formation of a 17-keto-steroid (Figure 4.34) (Sih *et al.*, 1967a,b, 1968).

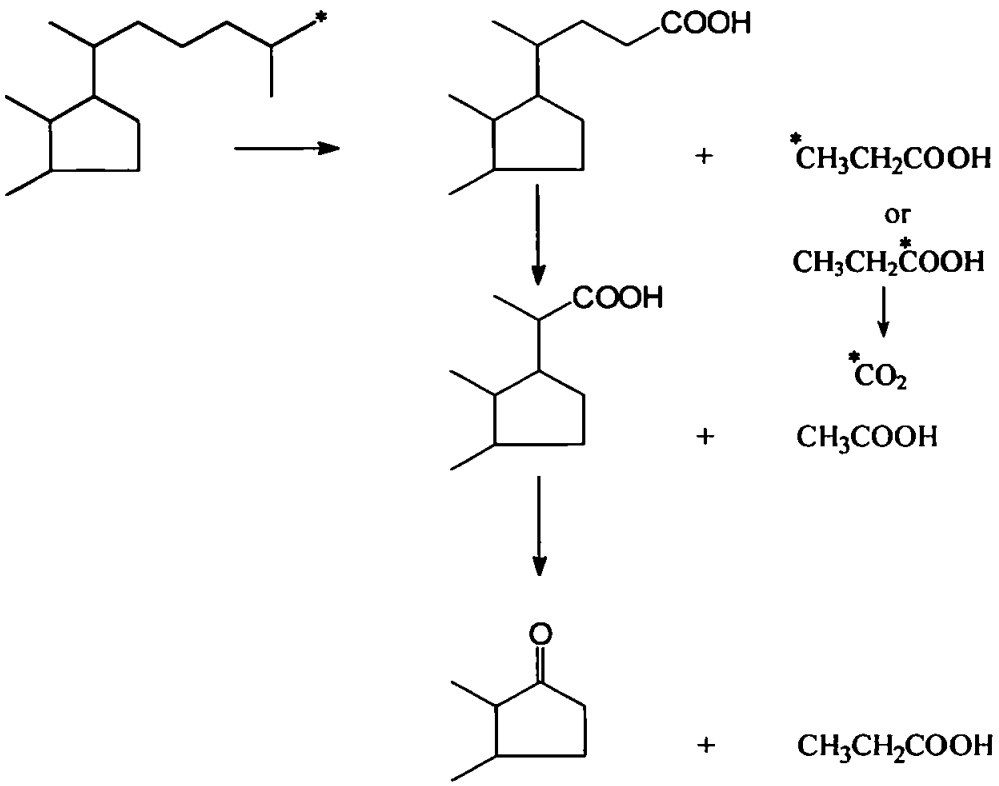
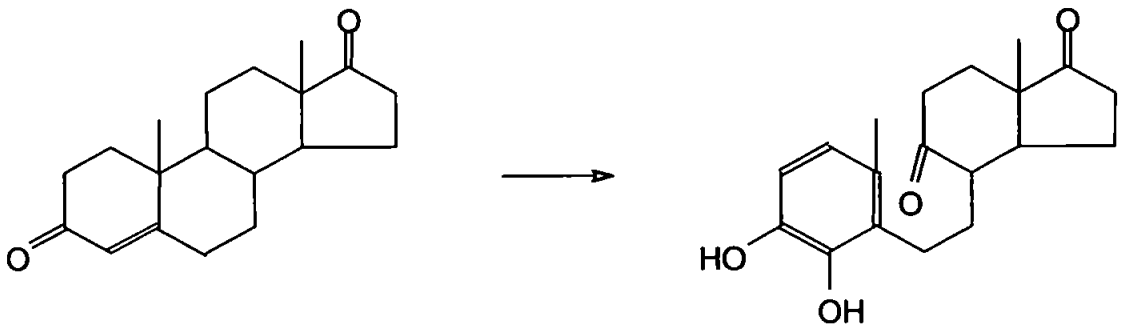


Figure 4.34 Stepwise bacterial cleavage of alkyl side chain resulting in the formation of a 17-keto-steroid (Sih *et al.*, 1967a,b, 1968). * denotes radiolabelled atom in the present study.

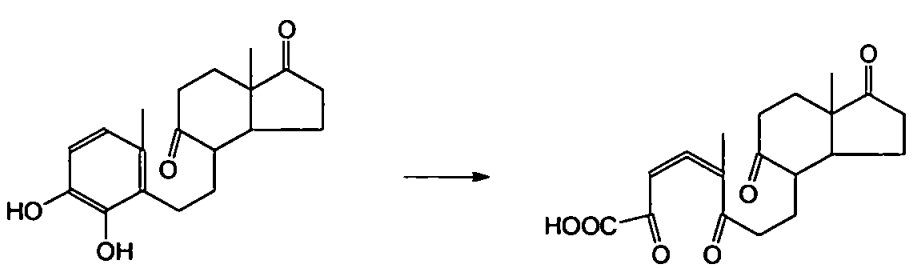
The biodegradation of the 4-^{14}C-cholesterol (**III**) used in the SCAS system resulted in a 29 % yield of ^{14}C carbon dioxide in the first 24 h. Such production is due to the A-ring opening releasing the C4 carbon atom. Sih *et al.* (1965a,) examined the **III** metabolite, androst-4-en-3,17-dione (**XXXXIX**) with the bacterium *N. restrictus* and identified that the inclusion of a α-hydroxyl at position 9 followed by oxidation to a keto group facilitated the cleavage of the C9-C10 bond opening the B-ring. Examination of the product determined that along with the B-ring opening the A-ring had undergone aromatisation to produce 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (**LXIX**) (Figure 4.35).



XXXXIX **LXIX**

Figure 4.35 Biodegradation of androst-4-en-3,17-dione (**XXXXIX**) to 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (**LXIX**) by the bacterium *N. restrictus* (Sih *et al.*, 1965a).

The **LXIX** under went rapid oxidative A-ring fission between C4-C5 (4 hydroxyl oxidation) resulting in the formation of 4(5),9(10)-diseco-androsta-1-(10),2-diene-3,5,9,17-tetraon-4-oic acid (**LXXXXII**) (Sih *et al.*, 1965b) (Figure 4.36).

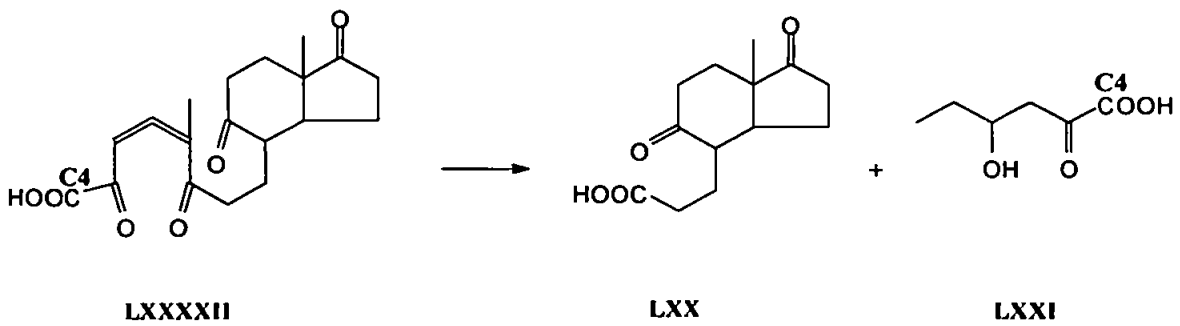


LXIX **LXXXXII**

Figure 4.36 A-ring rupture of **LXIX** leading to the diseco **LXXXXII** (Sih *et al.*, 1965b)

The 4(5),9(10)-diseco-androsta-1-(10),2-diene-3,5,9,17-tetraon-4-oic acid (**LXXXXII**) molecule has a carboxylic acid group on the C4 carbon. The molecule is further metabolised to release the C4 carbon as a short chain acid, 2-oxo-4-hydroxy-

caproic acid (**LXXI**), and 3aα-H-4α-[3'-propanoic acid]-7aβ-mehylhexahydro-1,5-indandione (**LXX**) (Figure 4.37)



LXXXXII **LXX** **LXXI**

Figure 4.37 The formation of **LXX** and **LXXI** from **LXXXXII** (C4 is highlighted) (Sih *et al.*, 1965b).

Coombe *et al.* (1966) studied the mechanism by which estrone (**IV**) underwent oxidative cleavage of the aromatic A-ring using *Nocardia sp.* The mechanism was shown to occur in a similar manner as androst-4-en-3,17-dione (**XXXXIX**) with the inclusion of a hydroxyl group in position 4 forming a catechol. The B-ring of **IV** did not, however, undergo 9α-hydroxylation and subsequent oxidation to cleave the C9-C10 bond (*i.e.* B-ring rupture). The 4-hydroxylation was the first step in the mechanism followed by oxidation resulting in C4-C5 bond cleavage. The resulting keto group at position 5 underwent reduction to form a diol that, upon oxidation formed a carboxylic acid facilitating C5-C10 bond cleavage. Thus, **IV** metabolism follows an A,B-ring fusion route. Afonso *et al.* (1966) reported that NCT (**LXXXI**) incubated with *Nocardia restrictus* yielded 8 % **IV** over 240 h. Thus, NCT (**LXXXI**) underwent β-oxidation of the side chain but both A and B-rings were conserved. The authors did not, however, report any other metabolites (*i.e.* remaining 92 % **LXXXI**) of the incubation such as 4,5 seco or 9(10) seco compounds (A-ring, B-ring rupture). These results suggest that the oxidative metabolism of steroids proceeds initially *via* side

chain cleavage. With the side chain removed the next step, B-ring or A-ring rupture, appears to be dependent on the nature of the A-ring present (*i.e.* aromatic or not). Therefore, herein it seems plausible that cholesterol (**III**) biodegradation proceeds *via* side chain cleavage followed by B-ring rupture, A-ring aromatisation and finally A-ring rupture. For the hypothesis to be proven and **LXXXI** production is in fact a metabolite of **III** biodegradation the side chain and B-ring must be conserved while the A-ring undergoes aromatisation

The percentage of ^{14}C carbon dioxide produced by both 4-^{14}C and 26-^{14}C-cholesterol (**III**) reported herein shows a slightly more rapid rate of mineralisation from 4-^{14}C **III** (29 % in 24 h and 40 % in 48 h, Figure 4.8) compared with 26-^{14}C **III** (20 % in 24 h and 29 % in 48 h, Figure 4.13). Therefore, it might be concluded that A-ring opening takes preference over side chain cleavage. For the A-ring of a steroid to be ruptured it has been shown that the B-ring is first ruptured followed by A-ring aromatisation (Nagasawa *et al.*, 1969). Thus if it could be proven that under certain circumstances the B-ring can also be conserved then NCT (**LXXXI**) synthesis might be expected. Sih *et al.* (1965c) suggested, however, that the increased yield of ^{14}C carbon dioxide from 4-^{14}C **III** observed in their studies may not actually mean that A-ring rupture and C4 carbon removal takes preference over side chain degradation. Sih and collaborators suggested that the mechanism of oxidative cleavage initially proceeds *via* side chain cleavage producing a C19 steroids followed by B-ring and A-ring rupture.

The aerobic metabolism of cholesterol (**III**) in the SCAS system was shown to be rapid with *ca* 50 % mineralisation occurring with 96 h. For the activity remaining in the mixed liquor 5 % was associated with the liquid fraction and 95 % with the SPM. The

liquid fraction, when extracted by SPE, showed that **III** and an unknown compound were both present. The efficiency of the SPE was not measured at this time. However, it is known that **III** has a high degree of retention to the SPE material (see Chapter 5 Tables 5.3 and 5.4). The retention of more polar compounds, although not determined, were likely to have a low affinity to the SPE and thus a high proportion would stay in solution. Therefore, polar compounds with relatively low log K_{ow} values resulting from A-ring rupture probably dominate the metabolites in the liquid fraction of the SCAS liquor.

The SPM contained the majority of activity remaining in the liquor and hence were more likely to be hydrophobic in nature. The extraction and rHPLC fractionation of the SPM showed that polar compounds present were minor products. For all SPM samples fractionated just two radiolabelled compounds were observed, the largest of which corresponded to unmetabolised cholesterol (**III**). The single metabolite seen, by rHPLC, eluted *ca* 45 seconds prior to **III** and was observed in all SPM extracts with the maximum concentration occurring after 48 h (Figure 4.11). The rGC analysis of the fractions taken proved that unmetabolised **III** was present but also showed an unidentified metabolite in the 48 h incubation fraction. Although not determined the metabolite was the only compound >LOD and by definition was the main metabolite (other than $^{14}CO_2$) as other compounds were < LOD.

The use of the SCAS system highlighted a number of problems with the technique. The amount of 4-^{14}C-cholesterol (**III**) added (49 KBq) was far too small to allow a full-scale examination of metabolites produced. For a more rigorous analytical approach at

least 500 KBq should be used. Therefore, although only a single metabolite was observed the examination does not truly reflect full aerobic metabolism.

The DA system, using 26-^{14}C-cholesterol (**III**) was used in an attempt to examine products of A-ring rupture, among other metabolites. Due to the reasons previously described the DA system could only run optimally for 48 h. Three systems were run simultaneously to study **III** biodegradation over 24 and 48 h as well as a time zero blank. To each unit 740 KBq 26-^{14}C **III** was added (equivalent to 151μg **III**), a 15-fold increase in activity used in the SCAS system. Unlike the SCAS system each individual DA unit was run for a specific incubation period after which time all the liquid and SPM were separated, extracted and analysed. The activity was determined in the liquid and SPM fractions and coupled with the activity due to mineralisation ($^{14}CO_2$) a mass balance calculation was determined (Figure 4.15). For the 24 h incubation study the mass balance determined was 82 %, 21 % and 1 % (SPM, $^{14}CO_2$ and liquid) compared with 85 %, 29 % and 2 % (SPM, $^{14}CO_2$ and liquid) for the 48 h study. The control gave 89 %, 2 % and <1 % in comparison. The slightly elevated total values encountered were probably due to pipetting inaccuracies.

The liquid samples extracted by SPE showed that *ca* 50 % of original activity in the liquid fraction was not retained and that that was retained was either cholesterol (**III**) or early eluting compounds. The SPE procedure was repeated with no further removal of activity from the liquid fraction and thus *ca* 50 % of activity (2000-4000 Bq) was not analysed further. The amount of activity in the liquid portion increased from 6 KBq (24 h) to 9 KBq (48 h), a 50 % increase. The $^{14}CO_2$ evolved increased from 152 KBq

(24 h) to 215 KBq (48 h) (*i.e.* a 42 % increase). The 26-^{14}C **III** added to the DA liquor would be associated with the SPM and thus the site for side chain cleavage.

The SPM contained 82 % (24 h) and 85 % (48 h) of the total activity added to the DA liquor compared with 89 % for the control. The SPM of the DA system was divided into 4 equal sub-fractions and analysed separately. Each SPM sub-fraction contained *ca* 100 KBq (> 30 fold increase in total activity when compared with solid samples taken from the SCAS). The SCAS solid extracts revealed only two peaks >LOD compared to between 12 and 20 individual peaks >LOD for the DA system. The fractions highlighted in Figure 4.18 were taken for rGC and GC-MS analysis..

Fraction 1 contained compounds of low affinity for the ODS HPLC column and were not identified by rGC or GC-MS. Fraction 2 contained two peaks, 3-4 % of the total SPM activity, and when analysed by rGC three peaks were determined (Figure 4.20). The smaller late eluting metabolite was found to be cholesterol (**III**) whereas the major peak was identified as cholest-3,5-diene (**LXXXIX**) ($M^{+\cdot}$ *m/z* 368) when analysed by GC-MS. The first, much smaller, eluting compound also had a $M^{+\cdot}$ of *m/z* 368 and although not fully identified was probably an isomer of **LXXXIX** such as cholest-2,4-diene (an authentic reference analyte of cholest-2,4-diene or any other diene could not be found commercially). Buckley *et al.* (1999) used sequential thermal desorption gas chromatography-mass spectrometry (TD-GC-MS), pyrolysis gas chromatography-mass spectrometry (Py-GC-MS) and GC-MS to examine residues of Egyptian mummies. The authors identified that cholesteryl ester compounds, related to cholesterol, subjected to TD-GC-MS underwent ester cleavage and dehydration to

form cholest-3,5-diene (**LXXXIX**). In the present study two cholestadienes were suggested to be metabolites of cholesterol, however, upon examination of Buckley and co-authors result it could be hypothesised that the dienes discovered herein could also be the result of thermal metabolism in the injector of the GC-MS. The cholestadienes in particular lack any functionality and as such it might be expected that these compounds would be retained by an ODS HPLC column longer than steroids such as cholesterol (**III**) that possess hydroxy functionality. Herein compounds eluting in fraction 3 (Figure 4.18) eluted *ca* 13 minutes before **III**. Thus, although not determined, the metabolites are probably not dienes but could be cholesteryl ester or cholesteryl sulphate compounds with a higher degree of functionality and hence less affinity with the ODS stationary phase.

Fraction 3, <5 % of the total activity, contained a number of small peaks <50 cps but unfortunately when analysed by rGC were <LOD. Fractions 4 and 5, *ca* 80 % of total activity, contained the majority of eluting compound with the majority of activity. Unaltered 26-^{14}C-cholesterol (**III**) made up the significant percentage of activity determined in both fractions (Figures 4.22 and 4.26). The aim of the research was to examine whether **III** can aromatise to form NCT (**LXXXI**). If **LXXXI** were a biodegradation product of **III** it would have been in either of the two fractions. **LXXXI** was not determined in either fraction from all 24 and 48 h solid samples when analysed by rGC and GC-MS. The inclusion of the rTLC step increased rGC sensitivity and aided confirmation that **LXXXI** was not present in the fractions. For **LXXXI** to be biosynthesised the **III** molecule must under go aromatisation of the A-ring with the loss of the angular methyl C19 and hydrogenation of the C5-C6 double bond. The side chain and B-ring must also be conserved. As previously mentioned androst-4-en-3,17-

dione (**XXXXIX**) underwent B-ring fusion prior to A-ring aromatisation (Sih *et al.*, 1965a). The side chain had previously undergone β-oxidation forming a keto group in position C17. The mechanism, therefore, of steroid degradation is either *via* side chain cleavage followed by A-ring aromatisation (and B-ring fusion) or *visa versa*. For NCT (**LXXXI**) to be produced from **III** requires the side chain to be conserved and the aromatisation of the A-ring without B-ring fusion. Sih and Wang, (1965c) studied 4-^{14}C-androst-4-en-3,17-dione (**XXXXIX**) and 4-^{14}C-17β-estradiol (**I**) metabolism with the bacterium *N. restrictus* and found that the aromatic A-ring of the **I** resulted in virtually 0 % metabolism over 120 h. The **XXXXIX**, however, had undergone A-ring fusion with total C4 carbon removal over 50 h and producing ^{14}C carbon dioxide. The significant differences between **XXXXIX** and **I** was the presence of the C19 angular methyl in **XXXXIX** and aromatic A-ring of the **I**. Thus, it might be inferred that either the aromatic A-ring inhibits metabolism or the C19 methyl plays a dominant role in biodegradation. For the laboratory synthesis of NCT (**LXXXI**) Kocovský and Baines (1994) showed that using 19-hydroxy-cholest-4-en-3-one (**LXXIII**) under relatively simple conditions produced a high yield of **LXXXI**. Within mammalian systems the production of sex hormones is *via* 19-hydroxy steroid intermediates (Devlin, 1997). Therefore, the C19 methyl had undergone hydroxylation, which allows the steroid to form an aromatic A-ring. Furthermore, Sih and Wang (1965c) showed that incubating **LXXIII** with *N. restrictus* resulted in the formation of estrone (**IV**) (8 %) (Figure 4.38). Thus, **LXXIII** had undergone side chain removal and A-ring aromatisation. However, the authors failed to mention whether any other by-products had also been produced. It is thus conceivable that if **LXXIII** were a metabolite of cholesterol (**III**) A-ring aromatisation and conservation of the side chain would produce NCT

(**LXXXI**). Subsequent side chain degradation would lead to estrone (**IV**) (Afonso *et al.*, 1966) (Figure 4.38).

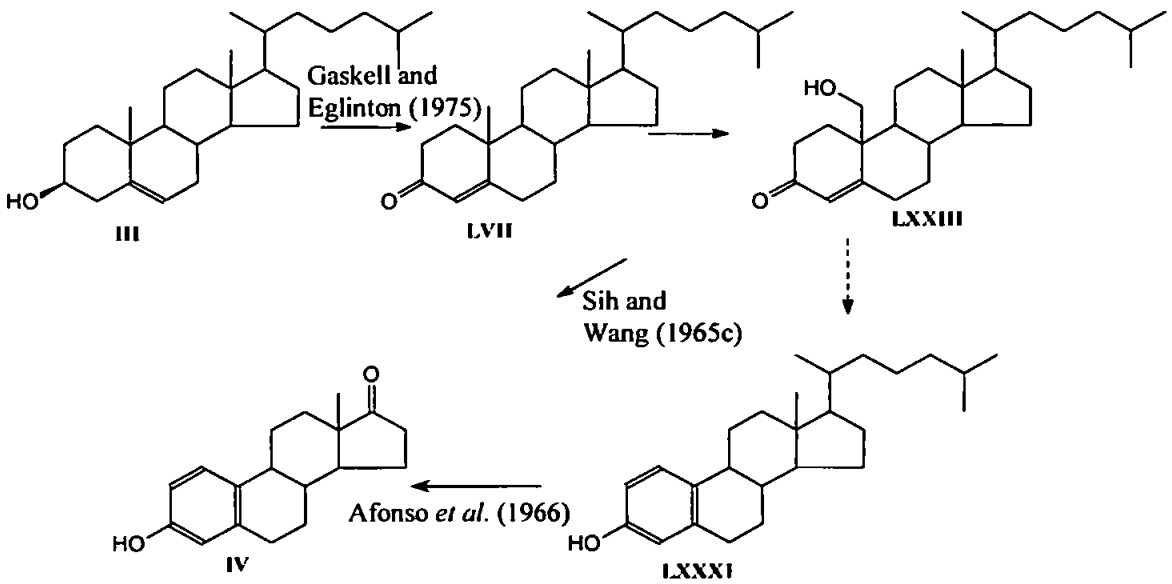
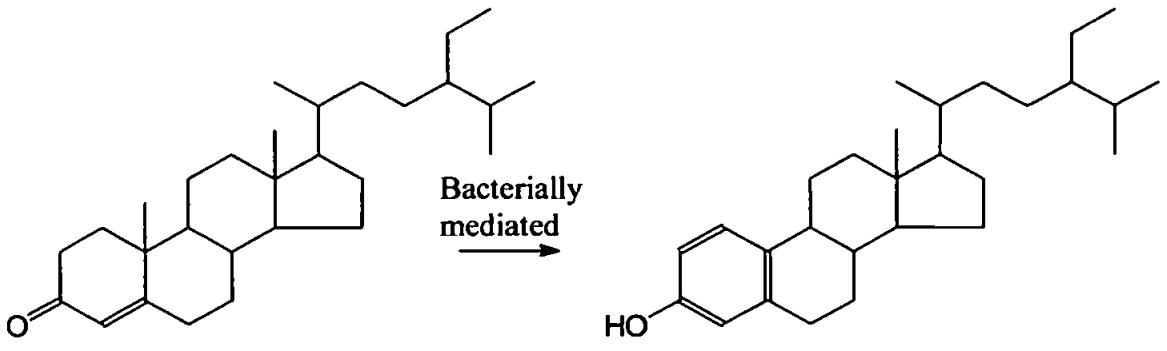


Figure 4.38 Biodegradation of **III** to produce **IV** and hypothesised route for **LXXXI** production *via* **LXXIII** intermediate.

The formulation of the 19-hydroxyl is believed to prevent 9α-hydroxylation, which in turn inhibits the B-ring from rupture (Bhattacharyya *et al.*, 1984). Bhattacharyya *et al.* (1984) also examined A-ring aromatisation on two 19-hydroxy-steroids (19-hydroxy-3β-acetoxy cholest-5-ene (**LXXV**) and 19-hydroxy-3β-acetoxy sitost-5-ene (**LXXVI**)) with a variety of bacteria and again identified estrone (**IV**) as a metabolite. Both Sih and Wang (1965c) and Bhattacharyya *et al.* (1984) used **LXXVI** species (*e.g.* 19-hydroxy-β-sitost-4-en-3-one (**LXXXXIV**)) to further demonstrate the ability of microorganisms to form **IV**. The use of sitosterone such as **LXXXXIV** could lead to the formation of the corresponding 19-norsitoster-1,3,5(10)-trien-3-ol (NST) (**LXXXVI**) (Figure 4.39).



LXXXXIV **LXXXVI**

Figure 4.39 Hypothetical bacterial pathway to NST *via* aromatisation of sitosterone A-ring under the influence of bacteria.

For either NCT (**LXXXI**) or NST (**LXXXVI**) to be produced during wastewater treatment there must either be a source of 19-hydroxysteroids *via* the influent) or *via in situ* biosynthesis. No data is currently available for excretion of 19-hydroxy-steroids such as 19-hydroxycholest-4-en-3-one (**LXXIII**) being excreted from the mammals or from *in situ* production *via* microorganisms. If **LXXIII** either entered STW in the influent or was synthesised *in situ* then the formation of **LXXXI** and analogues might be possible.

Four main products of cholesterol (**III**) metabolism were identified in HPLC fractions 4 and 5. Cholest-4-en-3-one (**LVII**) was identified as a metabolite, but was only found in two of the solid samples from 24 h. **LVII** is known to be a metabolite of **III** when incubated with sewage sludge under anaerobic conditions (Gaskell and Eglinton, 1975) and is known to be the first major metabolite of **III** during biodegradation (Bhattacharyya, *et al.*, 1984). Nagasawa *et al.* (1969) showed that **III** incubated with *Arthrobacter simplex* yielded **LVII**. Harvarth and Kramli (1948) also showed that *Azotobacter* produced **LVII** using **III** as a substrate. The formation of cholest-4-en-3-

one (**LVII**) maybe the first main step in B-ring rupture and subsequent A-ring aromatisation and rupture. Initially, for the B-ring to undergo rupture a 9α-hydroxyl group needs to be introduced (see previous). The enzyme responsible for hydroxylation, 9α-hydroxlase, was shown to be inactive with **LVII** but androst-4-en-3,17-dione (**XXXXIX**) was shown to be a good substrate (Chang and Sih, 1964). **LVII** has been shown to undergo β-oxidation of the side chain with many bacteria (Nagasawa *et al.*, 1969) to form **XXXXIX**. Dobson and Muir (1961) used the bacterium *N.restrictus* and **XXXXIX** substrate to produce androst-4-en-3,17-dione-9α-ol (**LXXXXIII**). The insertion of the 9α-hydroxyl group would thus allow oxidative cleavage of the B-ring followed by A-ring cleavage, previously described.

Two further unidentified products were discovered by rGC and using GC-MS and retention indices of the TMS ether derivative the $M^{+\cdot}$ were *m/z* 456 and 458. The $M^{+\cdot}$ of *m/z* 456 suggests a cholestadienol such as cholest-5,7-dien-3β-ol (**LXXXX**) (vitamin D precursor). Harvath and Kramli (1947) first described the dehydrogenation of cholesterol (**III**) under the influence of *Azotobacter* to form **LXXXX**. The GC-MS analysis of the fraction, although the retention times were in good agreement, the mass spectrum ($M^{+\cdot}$ was *m/z* 456) obtained did not agree (Figures 4.24 and 4.25). The sites of dehydrogenation in steroids have been reported at Δ^{1}, Δ^{4}, Δ^{7},$\Delta^{9(11)}$, Δ^{14} and Δ^{16} on the cyclopentanoperhydrophenanthrene ring system (Figure 4.40) (Charney and Herzog, 1967).

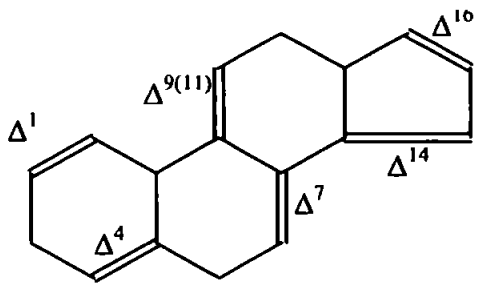
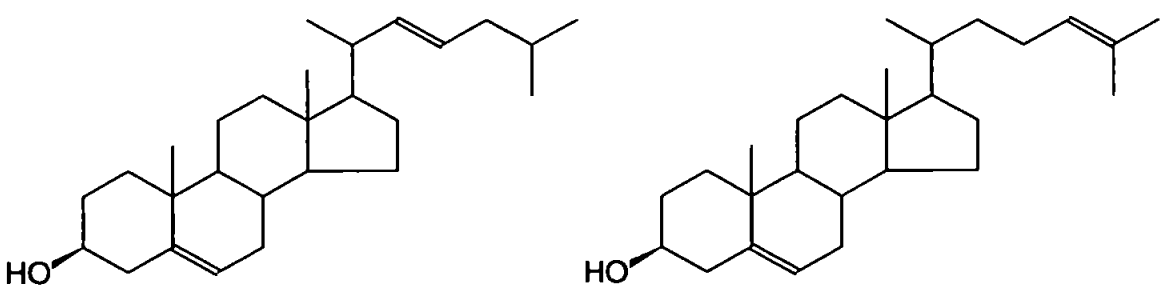


Figure 4.40 Sites of reported dehydrogenation on the cyclopentanoperhydrophenanthrene ring system

The dehydrogenation of the A-ring, 1- and 4-, usually occurs when the functional group at position 3 is a keto group (Charney and Herzog, 1967) and as such could not form the TMS ether derivative. Thus, for the $M^{+\cdot}$ of *m/z* 456, as the TMS ether at position 3 the dehydrogenation could occur at positions 7-, 9(11), 14- and 16- (assuming the original double bond at position 5 is conserved). The double bond could also be sited on the alkyl side chain (for example cholest-5,22-dien-3β-ol (**LXXXVII**) and cholest-5,24-dien-3β-ol (**LXXXVIII**) (Figure 4.41)). Unfortunately cholest-5,7-dien-3β-ol (**LXXXX**) was the only cholestadienol commercially available.



LXXXVII LXXXVIII

Figure 4.41 Structures of cholest-5,22-dien-3β-ol (**LXXXVII**) and cholest-5,24-dien-3β-ol (**LXXXVIII**).

Cholesterol (**III**) as the TMS ether derivative has a $M^{+\cdot}$ of 458. The radioactive compound with the $M^{+\cdot}$ of 458 did not co-elute with **III** and hence must be an isomer of **III**. The isomerisation of the Δ^5 double bond to the A-ring, most notably Δ^4 is also followed by oxidation of the β-hydroxyl to a keto group (Charney & Herzog, 1967). Therefore the Δ^5 double bond must have re-arranged to another region of the molecule.

4.5 Conclusion

Possible A-ring aromatisation cholesterol during sewage treatment to produce NCT was examined using laboratory anaerobic and aerobic technique.

The SPM samples from both oxic and anoxic regimes were solvent extracted efficiently.

The computed log K_{ow} value for NCT was > 9 (Chapter 3) and hence NCT would expected to be present in SPM extracts. The rGC and GC-MS analysis of *ca* 60 % of anaerobic SPM extracts and all aerobic extracts failed to indicate the production of ^{14}C NCT thus suggesting that either the cholesterol to NCT route was not possible, NCT does not bioaccumulate or NCT synthesised was <LOD for all analytical instruments used.

rHPLC analysis of the anaerobic SPM extracts indicated the existence of two metabolites of cholesterol in all samples examined. The two metabolites eluted before 4-^{14}C-cholesterol suggesting that all radiolabelled cholesterol had been biodegraded. The incubation period, 9 weeks, saw a steady reduction in ^{14}C products associated with the SPM. The rGC analysis, although limited by steroid burden in the extracts

showed that coprostan-3-one was the most abundent steroid. The rTLC also indicated the presence of coprostan-3-one over two solvent systems (NCT and coprostan-3-one co-elute).

The investigation of aerobic metabolism of cholesterol was performed using two individually ^{14}C labelled cholesterol, 26-^{14}C and 4-^{14}C. The rate of mineralisation of both species was comparable, 20-25 % in 24 h, with 4-^{14}C-cholesterol producing a slightly higher rate. Therefore, cholesterol undergoes both A-ring rupture and side chain cleavage. The SPM taken from the SCAS system contained one metabolite (other than the more abundant unmetabolised 4-^{14}C-cholesterol also present) when analysed by rHPLC. In comparison, the DA system contained 12-20 individual metabolites (unmetabolised 26-^{14}C-cholesterol was the most abundant species). The subsequent rGC and GC-MS of rHPLC fractions indicated the presence of cholest-3,5-diene and a possible isomer, however, upon closer examination of the rHPLC characteristics it seems unlikely that the cholestadienes were produced as a result of bacterially mediated metabolism but could in fact be the result of thermal decomposition of a much more polar compound such as cholesteryl sulphate. Among other metabolites were a dienol, an isomer of cholesterol and cholest-4-en-3-one. Thus cholesterol had undergone, apart from mineralisation, dehydration, dehydrogenation, isomerisation and oxidation of the hydroxyl functional group. The determination of >50 % of metabolites seen by rHPLC were not identified.

Chapter 5

Monitoring of selected estrogenic compounds and other sterols in STW effluents

5.1 Introduction

In spite of the recognised influence of effluents from some U.K. STW to produce 'estrogenic' effects on caged fish (*i.e.* increased vitellogenin production) demonstrated by the now famous studies of Sumpter and colleagues (Harris *et al.*, 1996) at the inception of this study, few chemical analyses of U.K. STW for estrogens (*e.g.* estrone (**IV**), 17β-estradiol (**I**)) had been published. During the present study Desbrow *et al.* (1998) published a detailed account of analyses of STW effluents from some of the STW studied by Harris *et al.* (1996). Nonetheless, there is an urgent need for further analysis of this type given the worldwide concern over the possible estrogenic effects of STW effluents (IEH, 1999). Therefore part of the current study into the origins and occurrence of estrogenic organic compounds in STW involved a detailed analytical study of two U.K. STW (Deephams and Harpenden). Those chosen were two STW effluents known to produce estrogenic effects on fish (*e.g.* Purden *et al.*, 1994; Harris *et al.*, 1996)

5.2 Sampling locations

STW effluent samples were taken from two sewage treatment works located at Deephams and Harpenden, Hertfordshire, throughout 1998 (Figure 5.1).

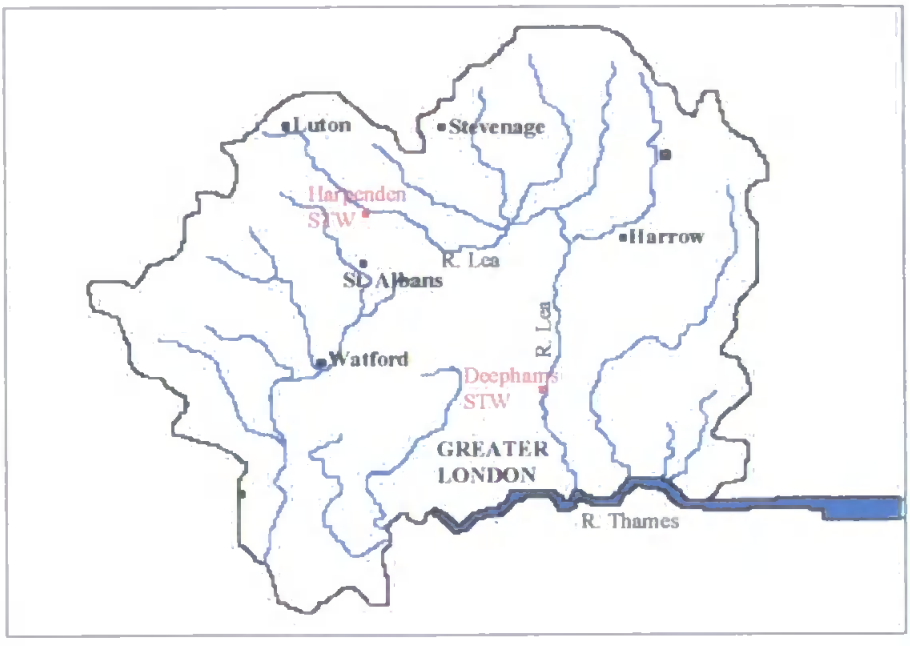


Figure 5.1 Site map of North London showing the River Lea* and the position of Harpenden and Deephams STW.

* Spelling of this river vary from Lea and Lee depending on the age of the cartographic source. For convenience, Lea is used in the thesis.

The date and times of sampling from both sites are shown in Table 5.1.

Sewage Treatment Works	Date	Local time sampled
Deephams	9th February, 1998	1430
Deephams	22nd April, 1998	1400
Deephams	13th May, 1998	1320-1420
Harpenden	20th May, 1998	1300-1320
Deephams	7th July, 1998	1110-1415
Harpenden	2nd August, 1998	0715-1630
Deephams	20th August, 1998	0930-1615

Table 5.1 Date and time effluent samples were taken from Deephams STW and Harpenden STW.

Samples were taken from the sampling site at *ca* 1400 h each day during times of dry weather in order to keep dilution effects to a minimum.

5.2.1 Deephams

The Deephams STW (Figure 5.2) is a diffused air activated sludge plant catering for a population of 796 000 people with a mean daily effluent flow of 1.6×10^8 L d^{-1} which is released into the river Lea (Environment Agency Report, 1997).

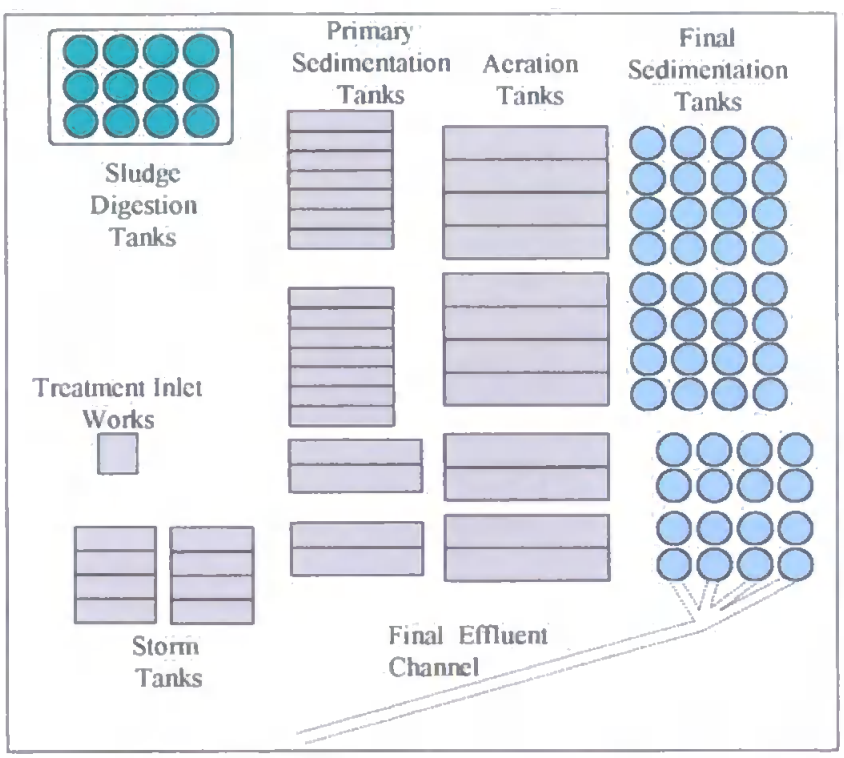


Figure 5.2 Schematic representation of the Deephams STW showing the final effluent channel where samples were collected.

5.2.2 Harpenden

Harpenden STW treats the sewage influent of a population of 31 200 people with a mean daily effluent flow of 8.25×10^6 L d^{-1} being released into the river Lea (Environment Agency Report, 1997). Treatment is by percolating filters and sand filters.

5.3 Method development

The aim of monitoring STW effluent was to assess the environmental concentration of NCT (**LXXXI**), if any, into the environment. Natural estrogens 17β-estradiol (**I**), estrone (**IV**) and 16α,17β-estriol (**V**) and the synthetic estrogen 17α-ethynylestradiol (**XIII**) were also to be examined.

The diverse physico-chemical nature of mono- to trifunctionalised steroids such as cholesterol (**III**) and 16α,17β-estriol (**V**) is reflected in their properties, such as their water solubilities and octanol-water partition coefficients (K_{ow}). Thus, mono-hydroxylated **III** is a much more hydrophobic compound than the tri-hydroxylated, **V**. As expected, **III** has a much higher log K_{ow} value than **V** (Table 5.2).

	log K_{ow}			Solubility (mg L^{-1})
Compound	**Syracuse**	**Med Chem**	**Experimentally Determined**	**Experimentally Determined**
Daidzein	2.55			
16α,17β-estriol	2.81		2.45†	13.25#
Estrone	3.43	3.38	3.13†	12.42#
17β-estradiol	3.94	3.78	4.01†	12.96#
17α-ethynylestradiol	4.12		3.67†	4.83#
Cholesterol	8.74			
Coprostanol	8.82			
NCT	9.23	9.35		0.000243*
Stigmasterol	9.43			
β-sitosterol	9.65			

Table 5.2 The calculated log K_{ow} using the Syracuse and Med Chem algorithms compared with experimentally determined values. The experimental determined solubility for a suite of estrogens and sterols are also given.

Data from Tabak *et al.* (1981). * Value obtained as a result of computed log K_{ow} value. † Data from Syracuse web site, http://esc.syrres.com/

Conversely, 16α,17β-estriol (**V**) has a high water solubility. This presents a potential problem for precise determination of a range of compounds encompassing relatively water soluble steroids (*e.g.* estrone (**IV**), 17β-estradiol (**I**), **V** and 17α-ethynylestradiol (**XIII**)) and much less soluble steroids (*e.g.* **III**, β-sitosterol (**XXXI**), coprostanol (**LXXII**) and NCT (**LXXXI**)) in the same sample. All of the compounds with extremes in chemical properties must be efficiently extracted from the host matrix water and solid particulate matter (SPM) in STW effluents and from sampling apparatus if reliable results are to be obtained. Largely for logistical and partly for economic reasons, the use of sample liquid-liquid extraction of aqueous samples has been replaced in many laboratories by the extraction of aqueous samples by elution through so-called Solid Phase Extraction (SPE) cartridges. A small pre-filter is used to remove solid material then the liquid sample is passed through a plastic cartridge containing a range of sorbent materials, such as octadecasilane (ODS). ODS is a relatively hydrophobic material and is widely used to retain hydorophobic compounds.

5.4 Solid phase extraction (SPE)

The efficiency of SPE ODS cartridges for the removal of hydrophobic steroids was examined in the present study by spiking the top of a cartridge with a mixture of ~100 ng steroid^{-1} cholesterol (**III**), β-sitosterol (**XXXI**) and NCT (**LXXXI**) in methanol, and drawing 10 L of distilled water through the cartridge. Removal of **III**, **XXXI** and **LXXXI** from the methanol onto ODS should be quite a severe test of the ODS interaction with the steroids and the efficiency should represent a worst case scenario since the extraction of these hydrophobic steroids from water would be expected to be higher. Extraction efficiencies are shown in Table 5.3.

Steroid	% sorption efficiency
Cholesterol (**III**)	$x = 90.2$, $\sigma_{n-1} = 9.4$, $n = 3$
β-sitosterol (**XXXI**)	$x = 88.9$, $\sigma_{n-1} = 7.5$, $n = 3$
NCT (**LXXXI**)	$x = 88$, $\sigma_{n-1} = 5.9$, $n = 3$

Table 5.3 Extraction efficiencies of steroids using an 5 g ODS SPE cartridge. Steroids, in methanol, were spiked onto the cartridge and 10 L water was drawn through. Steroids retained by the cartridge were eluted and determined by GC-MS. Values show extent of retention of each steroid

As expected, the ODS sorption of the hydrophobic, relatively water insoluble, steroids was high (*ca* 90 %). **III** and **XXXI** spiked into water at *ca* 10ng L^{-1} were removed with 77 and 120 % efficiency once a correction was made for loss of sterols due to sorption onto glass walls of the spiking vessel (Table 5.4).

Steroid	% sorption efficiency (from 10 L water)
Estrone (**IV**)	$x = 53.8$, $\sigma_{n-1} = 7.1$, $n=3$
17β-estradiol (**I**)	$x = 53.4$, $\sigma_{n-1} = 5.6$, $n=3$
16α,17β-estriol (**V**)	$x = 31.1$, $\sigma_{n-1} = 3.5$,$n=3$
Cholesterol (**III**)	$x = 77$, $\sigma_{n-1} = 7.8$, $n=3$
β-sitosterol (**XXXI**)	$x = 120$, $\sigma_{n-1} = 10.5$, $n=3$

Table 5.4 Total extraction efficiency using a 5 g ODS SPE cartridge for five key steroids from 10 L water after correction for sorption losses onto glassware[+].

[+] Losses of spiked compounds onto glassware can be appreciable. Thus, spiking experiments with insoluble compounds have to be conducted with care even when pre-dissolved in methanol or acetone. For example Zhou *et al* (1997) showed that ~ 70 % of the hydrophobic insecticide tefluthrin (K_{ow} similar to K_{ow} of cholesterol) rapidly sorbed to the walls of glass reaction tubes. This sorption may not occur when natural samples are extracted by SPE since a synergistic solubilisation of even hydrophobic compounds may occur and/or sorption onto natural organic solids may be predominant. Zhou *et al.* (1997) showed that in the presence of soil particles > 90 % of the tefluthrin added to water partitioned onto particulate matter and < 5 % was sorbed to the walls of the glass reaction vessels. In Chapter 4 radiolabelled cholesterol was added to a simulated STW using activated sludge. Fifteen minutes after addition of cholesterol 78 % had sorbed to the solids. These results further demonstrate the necessity of the examination of both the aqueous and solid samples.

The removal efficiencies of the more hydrophilic steroids, **IV**, **I** and **V** by SPE would be expected to be lower than those of **III**, **LXXXI** due to the increased functionality of the former. Indeed, this was observed, with values ranging between 31 to 54 % (Table 5.4). The extraction efficiency was improved to ~85 % in a recent study (Desbrow *et al.*, 1998) by use of two SPE cartridges in tandem. The use of two cartridges in the present study was prevented by the increase in back-pressure that reduced the flow of effluent through the cartridge beyond an acceptable level.

The difference in SPE efficiencies is reflected in the computed log K_{ow} values (Table 5.2). A plot of the log K_{ow} versus SPE efficiency shows a relatively linear relationship (Figure 5.3).

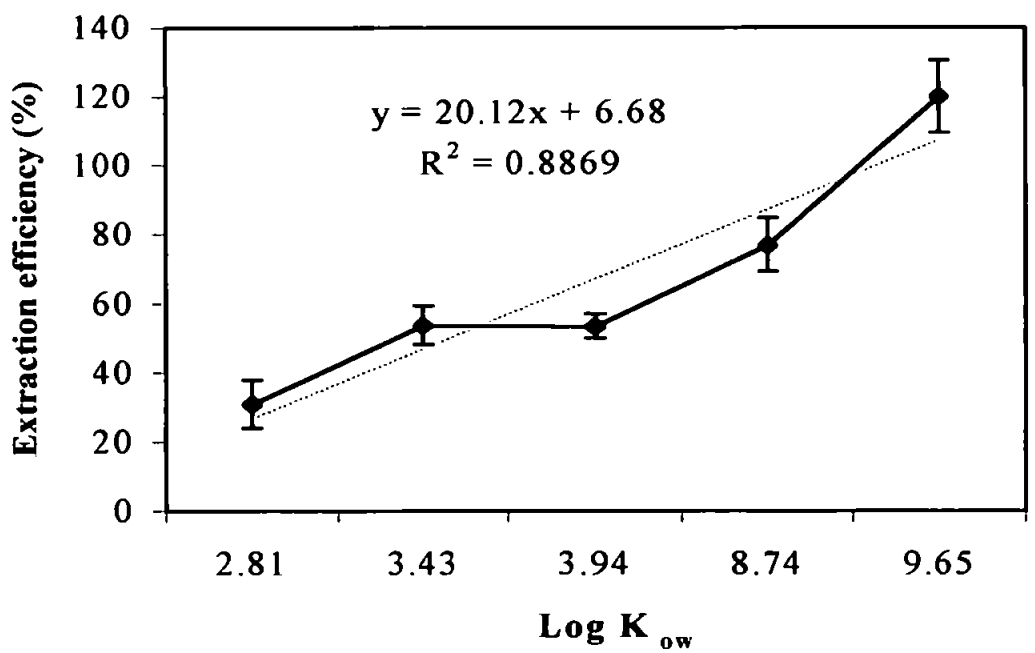


Figure 5.3 Correlation of extraction efficiency of five steroids from water by an ODS SPE cartridge with log K_{ow}.

The efficiencies of 30-100 % were deemed acceptable for the present study.

5.5 Extraction Efficiency of Solid Samples

In order to estimate the extraction efficiency of solid samples, a homogenised sediment sample was spiked with cholesterol (**III**) and NCT (**LXXXI**), (*ca* 100 ng per steroid) and dried at 40° C before extraction by ultrasonication for 15 minutes with methanol and DCM. Two glass fibre filters (GFC), used in the SPE pre-filters, were also spiked with **LXXII**, **III**, stigmasterol (**LXVI**) and **XXXI** (*ca* 100 ng per steroid) and extracted by solvent ultrasonication. The results of the extraction efficiency are shown in Figure 5.4.

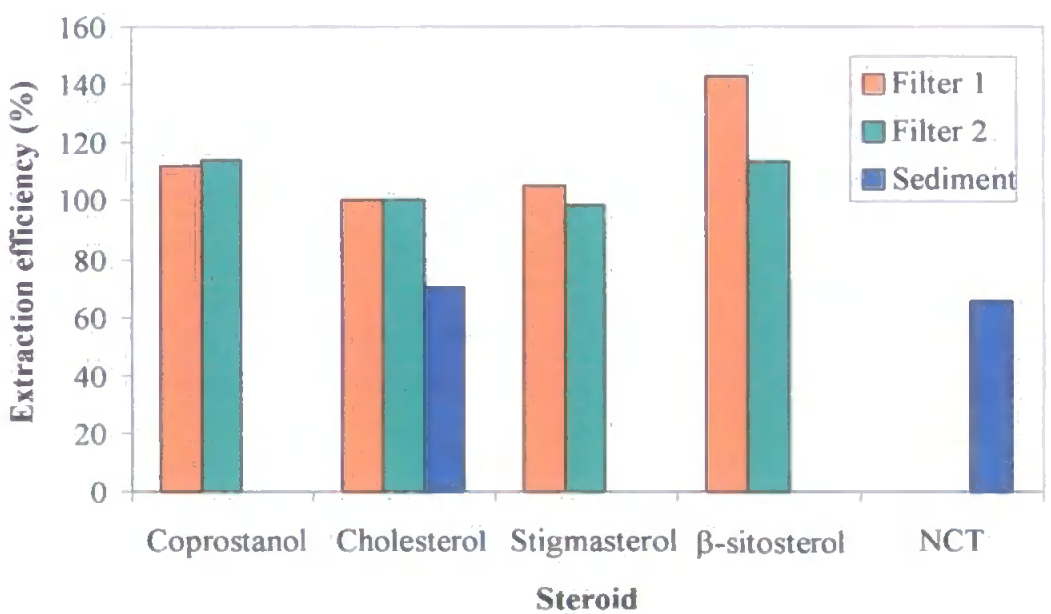


Figure 5.4 Extraction efficiencies of steroids added to GFC filters and a homogenised sediment (n = 3).

Figure 5.4 shows that, as expected, the GFC filter retained none of the sterols examined after extraction by ultrasonication with methanol/DCM. Cholesterol (**III**) and NCT (**LXXXI**) were recovered from the spiked sediment with 70 and 65 %

efficiency respectively, however, sediment is not an ideal matrix for modeling SPM encountered in sewage effluent. Nonetheless these results do indicate that the use of ultrasonication with methanol and DCM for extracting free steroids from filters or sediment medium was adequate.

5.6 HPLC fractionation

After extraction by SPE, effluent samples were fractionated by HPLC. Separation was achieved using a 25 cm x 4.6 mm *i.d.* ODS2 column. A methanol gradient, at a constant 1 mL min^{-1} flow rate, was used to elute compounds from the column. The amount of methanol initially used was 40 % at 0-3 minutes increasing linearly to 100 % after 30 minutes which was held for 10 minute before returning back to 40 % methanol. These were the conditions used by Desbrow *et al.* (1998) in their pioneering study of estrogens in STW effluents. Detection was by UV spectroscopy at a wavelength of 210 nm. Pure authentic analytes (*ca* 500 ng μL^{-1} in methanol), were used to establish the HPLC retention times of the individual compounds under these conditions. Estrone (**IV**) and 17β-estradiol (**I**) eluted at ~27 minutes. However, 16α,17β-estriol (**V**), eluted at ~3 minutes indicating that under these conditions this estrogen is barely retained. Probably for this reason previous studies have not measured the **V** burden of STW effluents (*e.g.* Desbrow *et al.*, 1998). The initial methanol percentage in the HPLC eluent was thus reduced from 40 % to 10 % in the present study. Table 5.5 shows retention times of compounds under investigation under the latter conditions, in which **V** is sufficiently retained for an improved fractionation.

Steroid	Retention time (minutes)	HLPC fraction collection time range (minutes)
16α,17β-estriol (**V**)	22	21.5-23
Estrone (**IV**)	26	24.5-26, 26-27.5
17β-estradiol (**I**)	26	24.5-26, 26-27.5
17α-ethynylestradiol (**XIII**)	26	24.5-26, 26-27.5
NCT (**LXXXI**)	37	33-40
Cholesterol (**III**)	38	33-40

Table 5.5 HPLC retention times and fraction collection retention time range for the major compounds under investigation.

HPLC fractions were collected every 90 seconds from 0 to 33 minutes and a final much larger fraction, was collected between 33-40 minutes which contained all the monofunctional sterols (Figure 5.5 and Table 5.5).

Before the examination of the effluent extracts, a methanol procedural blank was examined. Using the retention times in Table 5.5, fractions were taken within ± 1 minute of the retention time of each authentic compound to evaluate possible contamination. The procedural blank fractions were treated in the same manner as effluent fractions.

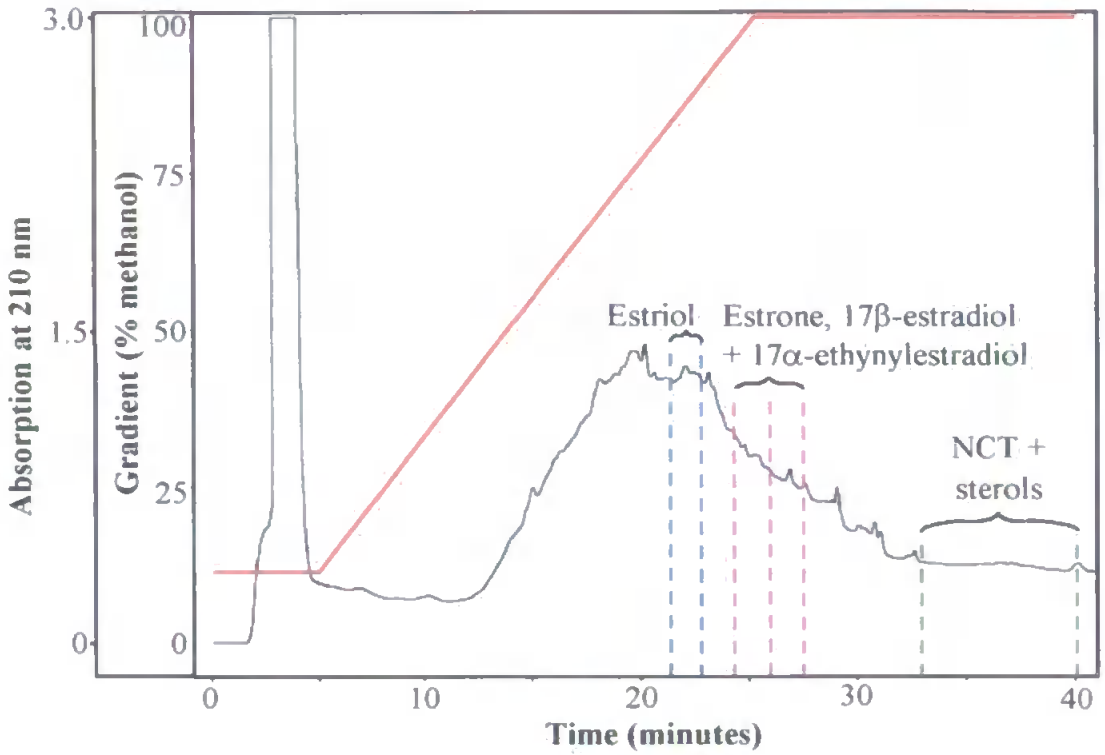


Figure 5.5 Typical HPLC chromatogram of a STW effluent extract using the revised gradient method. The main fractions of interest are highlighted.

Figure 5.5 shows that between 2 and 5 minutes (10 % methanol) there was a saturated signal due to elution of highly polar compounds having little affinity for the ODS stationary phase. The majority of compounds absorbing at 210 nm eluted between 13 to 33 minutes.

5.6.1 GC-MS response factors and retention indices

Prior to analysis of the sewage fractions the response factors of the authentic analytes were determined relative to 1,4-^{2}H-17β-estradiol (**I**) as the TMS ether derivative (using Σ *m/z* 418 ($M^{+\cdot}$) and 287). The results are shown in Table 5.6 along with the major ions used for the analytes (in summation where more than one major ion was used). The retention indices determined on HP5-MS phase are also shown.

Compounds	Response Factor (relative to 1,4-^{2}H-17β-estradiol)	Σ Ions Used (*m/z*)	Retention Indices (on HP5-MS column)
Estrone TMS	x = 1.26, σ_{n-1} = 0.16	**342**	2636 ± 0.6, n = 6
17β-estradiol *bis* TMS	x = 1	285, **416**	2676 ± 0.6, n = 12
16α,17β-estriol *tris* TMS	x = 0.73, σ_{n-1} = 0.09	386, 414, **504**	2875 ± 0.8, n = 3
Daidzein *bis* TMS	x = 4.01, σ_{n-1} = 0.7	383, **398**	
Coprostanol TMS	x = 3.72, σ_{n-1} = 0.3	370	3089 ± 1, n = 3
Cholesterol TMS	x = 0.95, σ_{n-1} = 0.09	368, **458**	3193 ± 0, n = 4
NCT TMS	x = 2.43, σ_{n-1} = 0.2	**440**	3213 ± 1.27, n = 4
Cholest-4-en-3-one	x = 60, σ_{n-1} = 7	124, **384**	3289 ± 0.6, n = 3
Stigmasterol TMS	x = 3.72, σ_{n-1} = 0.4	394, **484**	3425 ± 1, n = 3
Cholest-1,4-dien-3-one	x = 35.2, σ_{n-1} = 1.1	122	3416 ± 0.6, n = 3
β-sitosterol TMS	x = 5.6, σ_{n-1} = 0.5	396, **486**	3488 ± 1.5, n = 3

Table 5.6 Response factors (relative to 1,4-^{2}H-**I**) and retention indices on HP5-MS of a suite of authentic reference compounds. $M^{+\cdot}$ ions are given in bold.

The limit of detection (LOD), defined as signal to noise ratio greater than 3 was 0.1 ng L^{-1} for estrone (**IV**), 17β-estradiol (**I**) and 17α-ethynylestradiol (**XIII**) and 0.05 ng L^{-1} for 16α,17β-estriol (**V**) (all as the *bis* or *tris* TMS ether derivatives). The limit of detection for the other sterols was *ca* 1 ng L^{-1}.

The external standard, 1,4-^{2}H-17β-estradiol (**I**), was initially intended to be added to the STW effluents prior to SPE extraction in order to be used as an internal standard. However, GC-MS examination of 1,4-^{2}H-**I** (as the *bis* TMS ether derivative), indicated that it was not separated from the *bis* TMS ether of **I** and that the ions used by previous workers (*e.g.* Desbrow *et al.*, 1998) to monitor **I** bis TMS ether (*m/z* 416 ($M^{+\cdot}$) and 285) were also present in the 1,4-^{2}H-**I** bis TMS ether (Figure 5.6). Selected

ion monitoring of the 1,4-^{2}H **I** showed that ions *m/z* 418 + 287 and *m/z* 416 + 285 were all present in commercial 1,4-^{2}H-**I** (Figure 5.7). The area percentage response of ions *m/z* 416 and 285 compared with *m/z* 418 and 287 was *ca* 3 % (Figure 5.7).

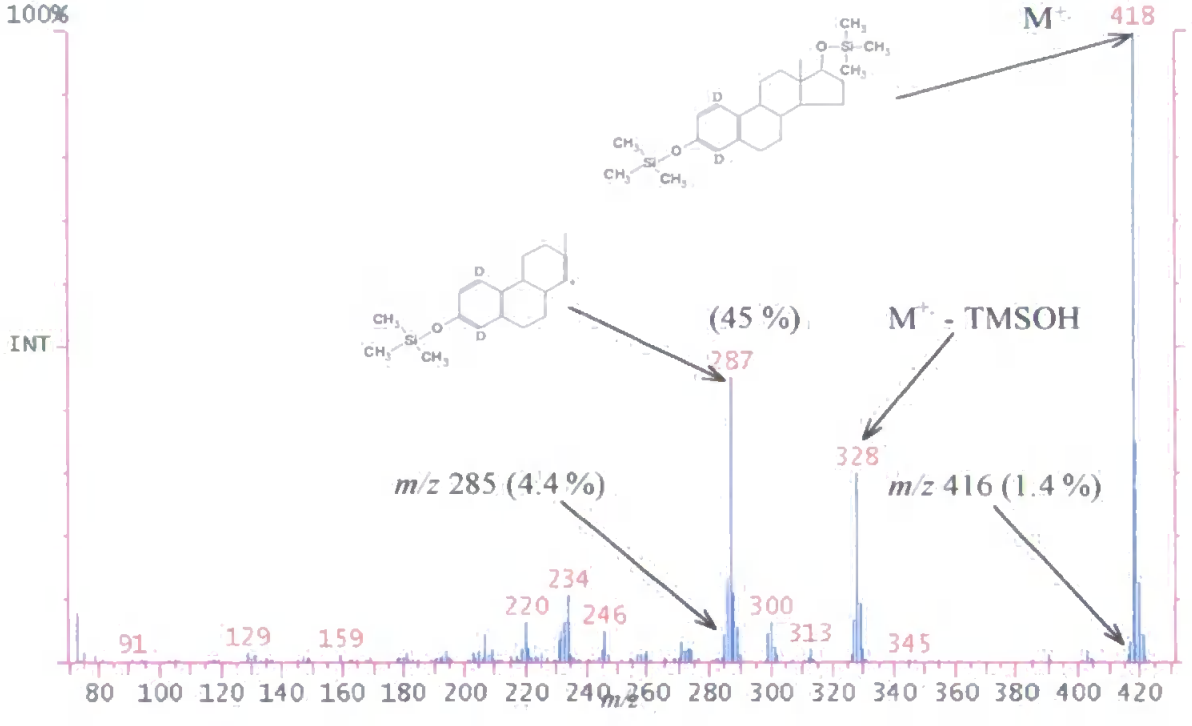


Figure 5.6 Mass spectrum of 1,4-^{2}H-**I** (as the *bis* TMS ether derivative).

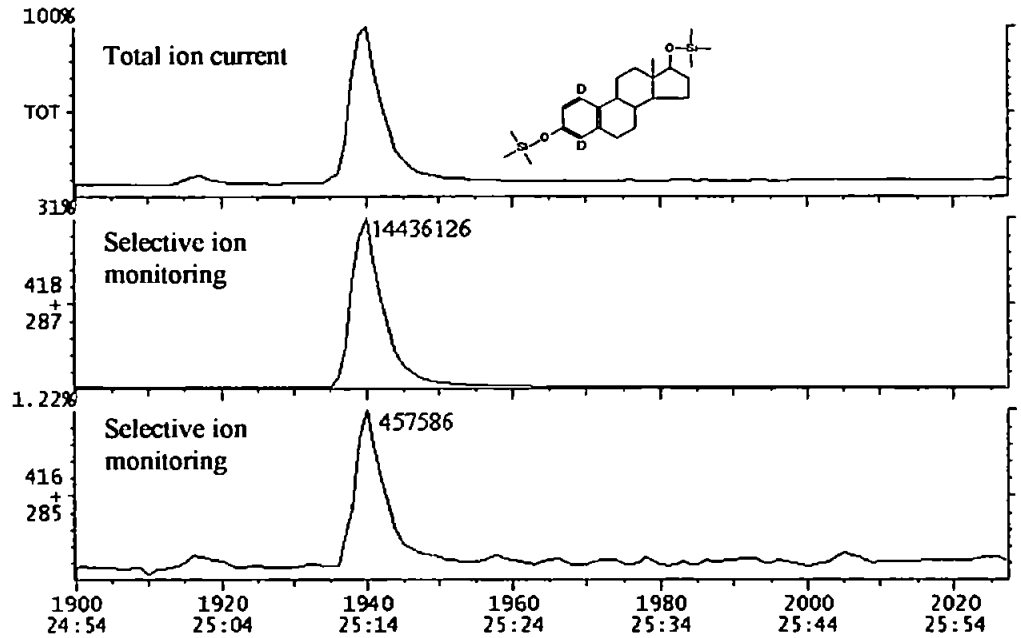


Figure 5.7 Selected ion monitoring of *m/z* 416 + 285 and 418 + 287 in commercially available 1,4-^{2}H-**I**.

The occurrence of ions *m/z* 416 and 285 might be due the presence of non-deuterated 17β-estradiol (**I**) in the deuterated compound. However, examination of the ion ratios suggests this is not the case (Table 5.7).

m/z	**Abundance compared to the $M^{+\cdot}$ 418 (%)**	**Ratio**	
418	100	418/287	2.2
287	45		
416	1.4	416/285	0.33
285	4.4		

Table 5.7 The relative abundances of ions used to monitor **I** and deuterated **I** in the electron impact mass spectrum of 1,4-^{2}H-**I**.

If ions *m/z* 416 and 285 were due to the presence of 17β-estradiol (**I**) in the deuterated analogue the ratio of *m/z* 416/285 would be expected to be similar to that of *m/z*

418/287. Such was not the case (Table 5.7). The addition of the 1,4-^{2}H-**I** to the STW effluent before SPE and monitoring *via* GC-MS *m/z* 416 + 285, would therefore result in an over estimation of **I** in the effluent. Thus either 1,4-^{2}H-**I** could be added as an internal standard and a correction applied to compensate for *m/z* 416 + 285 or 1,4-^{2}H-**I** could be used as an external standard for all fraction prior to GC-MS analysis except that containing 17β-estradiol (**I**). To determine **I** concentrations linear regression of 1,4-^{2}H-**I** could be used. It is possible that previous studies employing this method (*e.g.* Desbrow *et al.*, 1998) have over estimated the **I** burden in STW effluents as no mention of internal standard correction was reported.

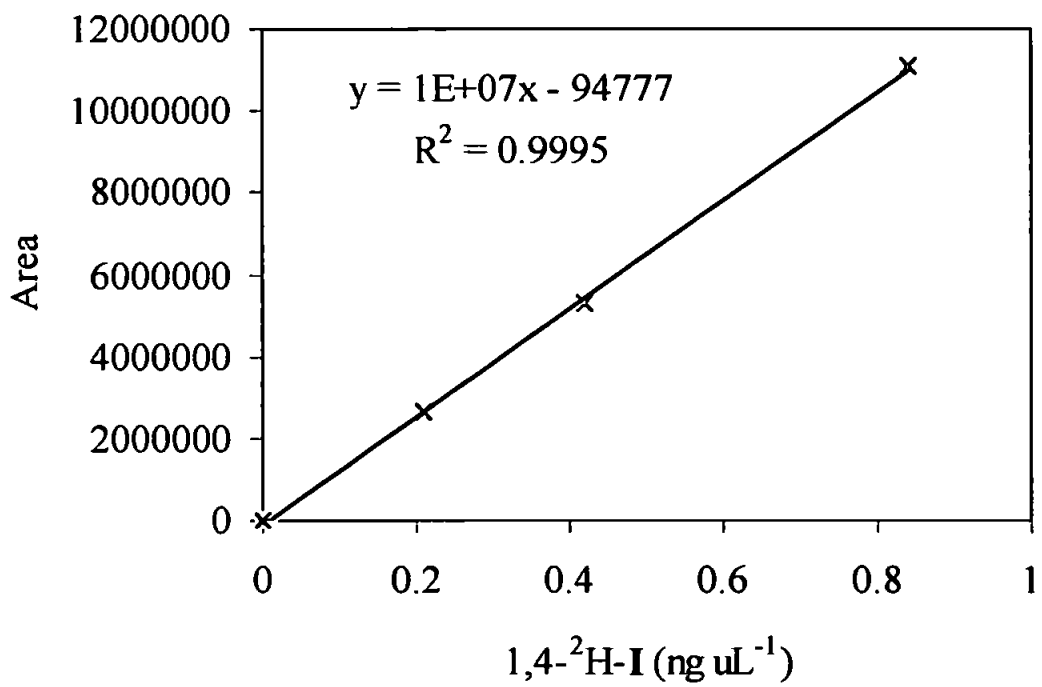


Figure 5.8 Linear regression of 1,4-^{2}H-**I** concentration plotted against peak area used to determine **I** concentrations in extracted treated sewage effluent (error bars = ± $1\sigma_{n-1}$, n = 3). Error are too small to be visible on this scale.

For this reason the author decided to use the deuterated standard as an external standard being added to all HPLC fractions except fractions containing **I**, prior to

derivatisation with BSTFA and GC-MS analysis, and all analytes were measured relative to the deuterated compound. For determinations of **I** concentrations, a series of 1,4-^{2}H-**I** standards were analysed by GC-MS immediately prior to GC-MS examinations of the 17β-estradiol (**I**) fraction (Figure 5.8). The values obtained were used to calculate **I** values.

5.7 Results

The concentrations of dissolved estrone (**IV**) measured in Deephams STW effluent varied from between 0.2-3.4 ng L^{-1} between February and September 1998 with the highest concentrations recorded in February and July (Figure 5.9; see Appendix A for tabulated results). The concentrations of 17β-estradiol (**I**) determined were usually a little lower than for **IV**, typically 0.2-1 ng L^{-1} (Figure 5.9). The highest concentration of **I** measured (July, 1 ng L^{-1}) coincided with a concentration of **IV** of 3.1 ng L^{-1} (see Figure 5.9). Figure 5.10 shows GC-MS chromatogram monitoring of **IV** and **I** (as TMS ether derivatives), mass spectra and co-injection analysis.

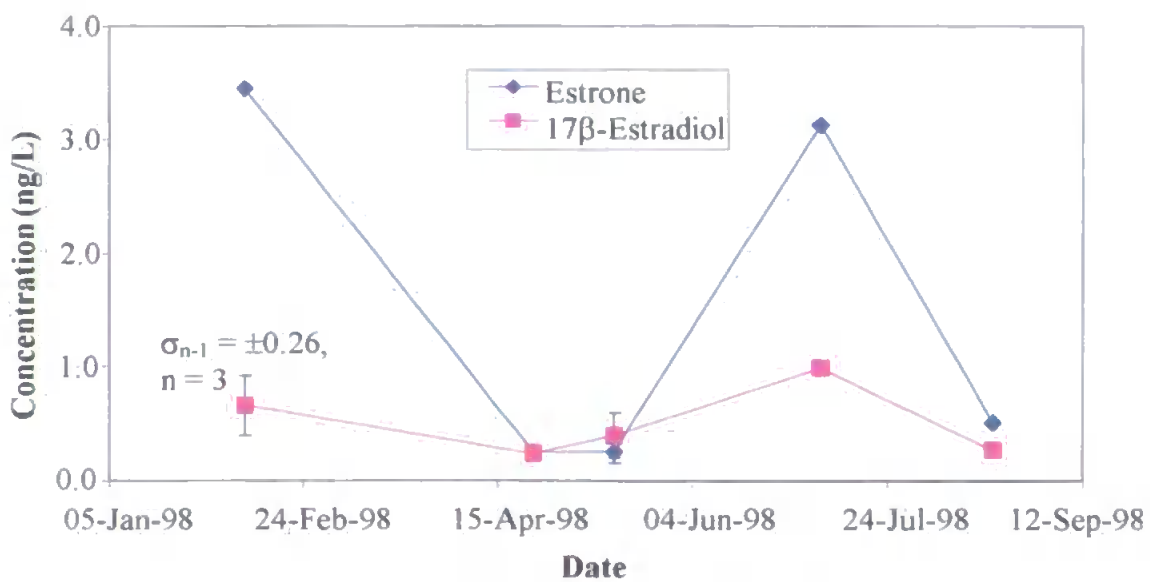


Figure 5.9 The concentrations of estrone (**IV**) and 17β-estradiol (**I**) determined in effluents from Deephams STWs throughout 1998.

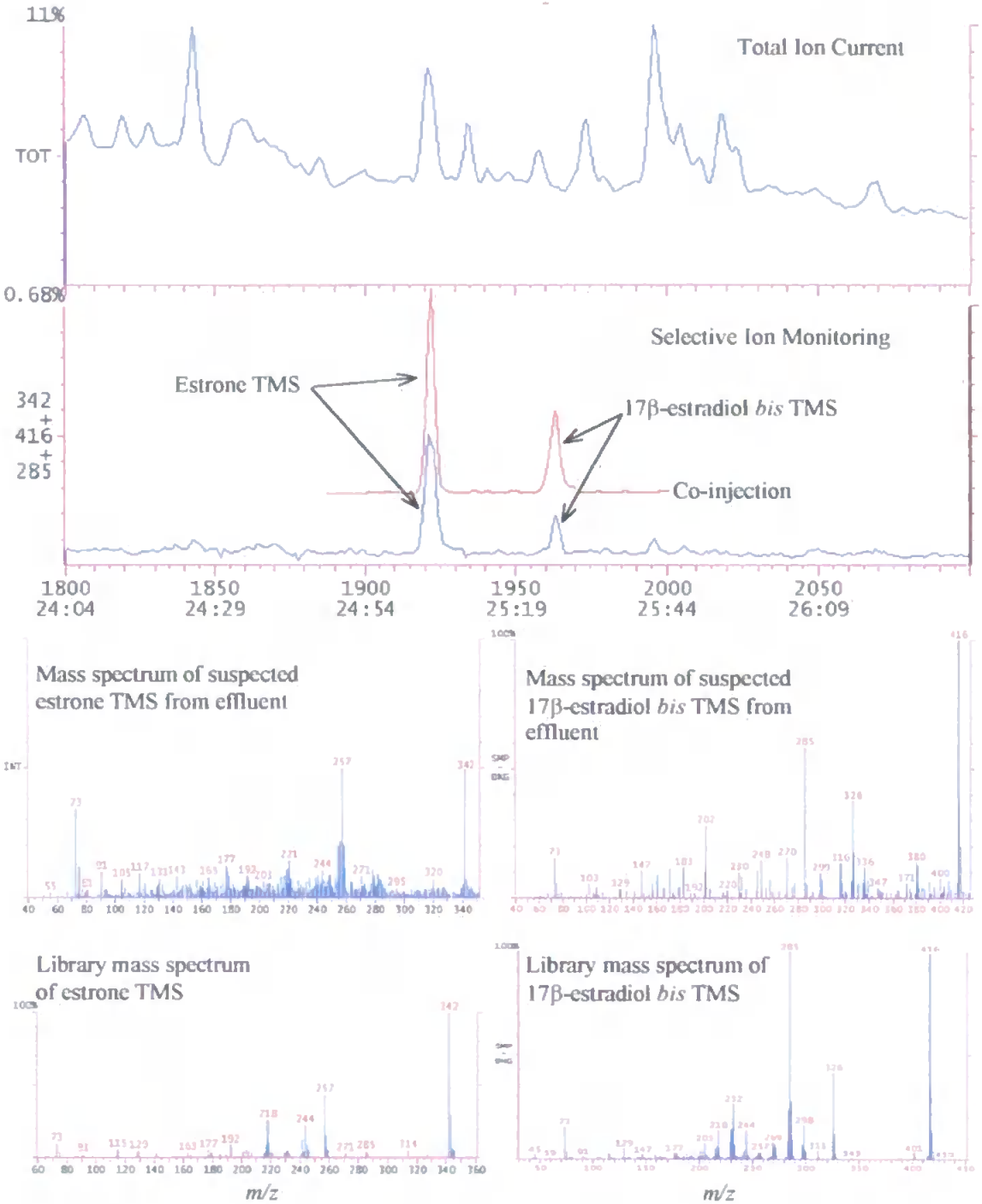


Figure 5.10 GC-MS analysis of Deephams STW effluent taken at 1415 h 7th July 1998 (HPLC fraction 24.5 – 27.5 mins) showing the presence of estrone (**IV**) and 17β-estradiol (**I**), as the TMS ether derivatives. Library mass spectra and co-injection experiments confirm the presence of **IV** and **I** in the effluent.

In order to assess the daily output of estrone (**IV**) and 17β-estradiol (**I**) released into the environment a time series of samples was taken between 09:15 and 16:30 every 45 minutes on 20th August, 1998 (Figure 5.11; see Appendix A for tabulated data). **IV** again tended to be higher in concentration than **I**. **IV** values ranged between 0.1-1.2 ng L^{-1} while the concentration of **I** ranged between <0.1 (LOD)-0.5 ng L^{-1}. Polynomial regression lines were fitted to the **IV** and **I** data. The regression coefficient (R^2) was low for the **I** (0.281). For the **IV** data set the polynomial fit was greater (0.682). The general trend lines indicate that the concentration of **IV** was significantly increasing after 1530 h. The concentration of **I**, however, generally reduced with time with a slight upturn near the end of the data set (Figure 5.11). Figure 5.11 shows how variable the concentrations of **I** (5 fold difference) and **IV** (6 fold difference) in the effluent can be over a short, seven h, period of time.

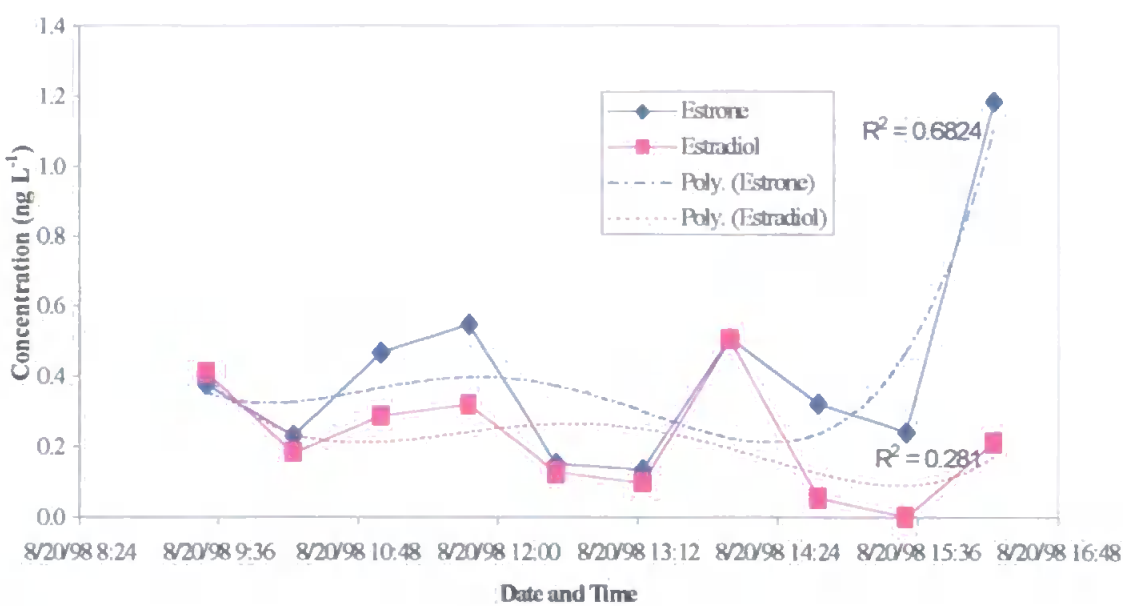


Figure 5.11 Concentrations of estrone (**IV**) and 17β-estradiol (**I**) over the course of 0930 – 1615 h, from Deephams STW effluent, 8th August 1998. Samples were taken at 45 minute intervals. Trend lines fitted to both data sets indicate an increase of both **IV** and **I** after 1530.

Neither 17α-ethynylestradiol (**XIII**) or the hypothesised 17β-estradiol (**I**) metabolite, NCT (**LXXXI**), were not detected in any aqueous effluent samples from Deephams STW, whereas the sterols, coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**) were consistently observed in all aqueous samples taken (Figure 5.12 shows a typical total ion current GC-MS chromatogram). The variations in concentrations of **LXXII**, **III**, **LXVI** and **XXXI** measured throughout the year and for the time series data are shown in Figures 5.13 and 5.14.

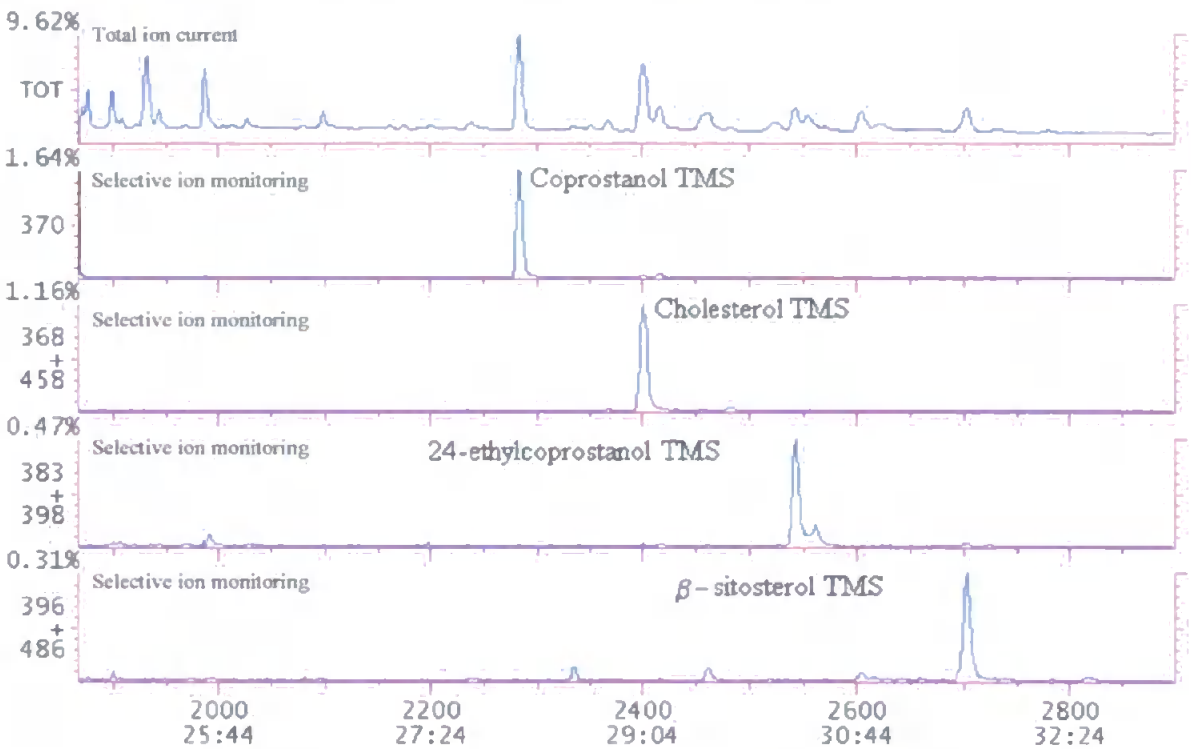


Figure 5.12 Partial GC-MS total ion current and selective ion monitoring chromatograms of four major sterols, as the TMS ether derivatives, determined from an aqueous effluent sample taken from Deephams STW at 1400 h 22nd April 1998.

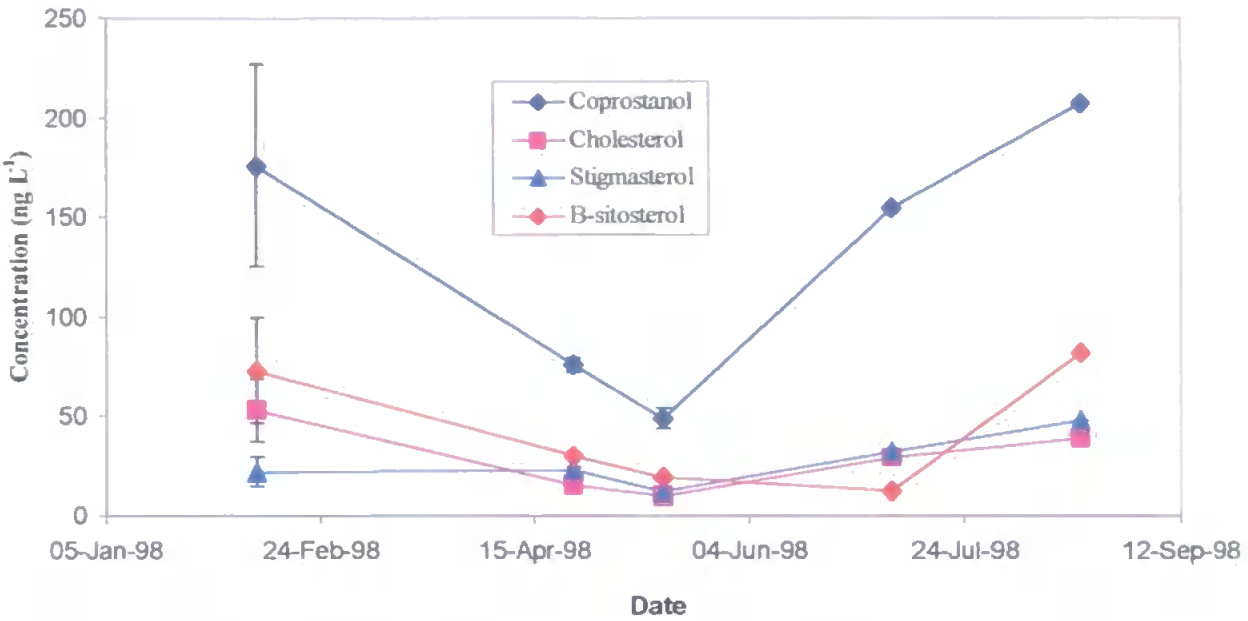


Figure 5.13 Concentrations of 4 main sterols measured from the Deephams STW throughout 1998.

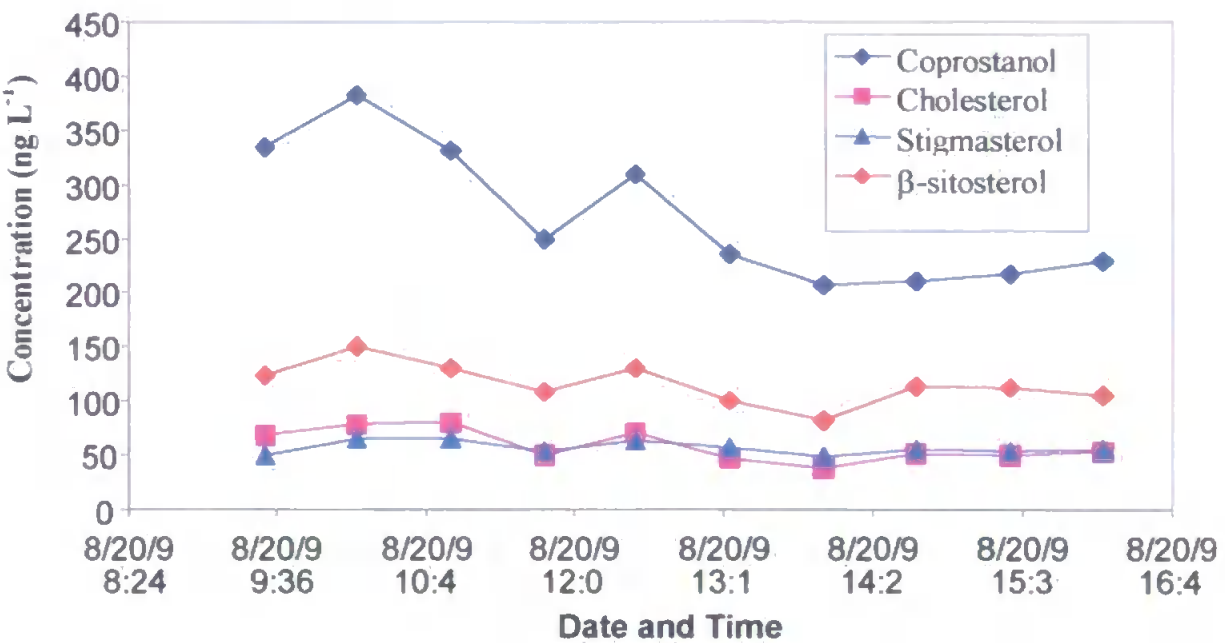


Figure 5.14 Concentration variablity of 4 main sterols measured during a 7 h time series (20th August, 1998) from the Deephams STW effluent.

Coprostanol (**LXXII**), a common intestinal mammalian metabolite of cholesterol (**III**) was detected in all samples at concentrations ranging between 50-383 ng L^{-1}. The

levels of **III** determined were almost an order of magnitude less with concentrations ranging between 10-80 ng L^{-1}. The levels of β-sitosterol (**XXXI**) were consistently higher than that of **III**, with concentrations ranging between 12-150 ng L^{-1}. Stigmasterol (**LXVI**) concentrations paralleled those of **III** with concentrations ranging between 12-66 ng L^{-1}. The sterone, cholest-4-en-3-one (**LVII**), was observed only once (7th August) with a concentration of *ca* 20 ng L^{-1} (Figure 5.15). The phytoestrogen, daidzein (**VIII**), was also observed (7th August) with a concentration of *ca* 3 ng L^{-1} (Figure 5.16). However, the analytical protocol was not optimised for the recovery of daidzein.

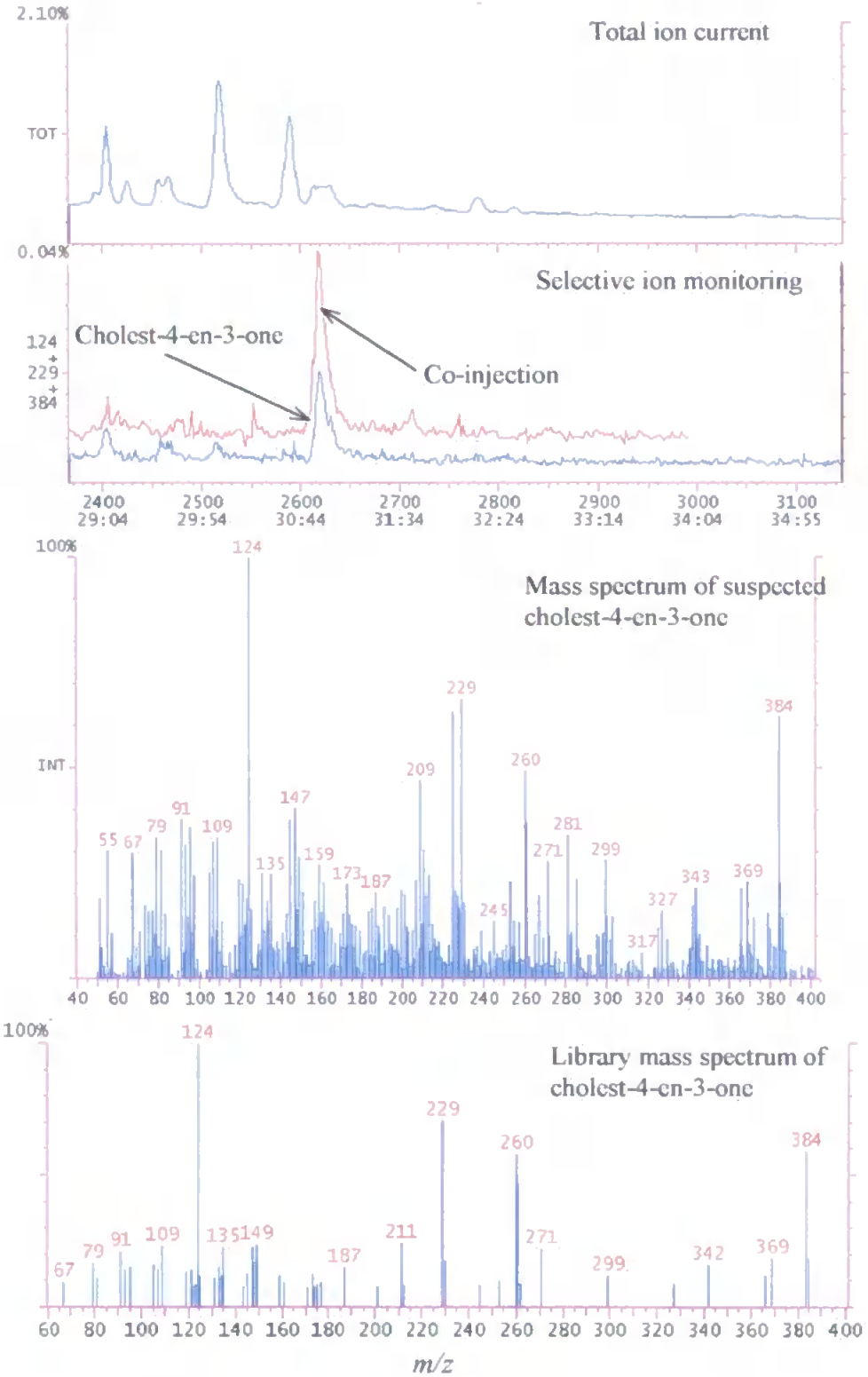


Figure 5.15 GC-MS analysis of Deephams STW effluent SPM taken at 1415 h 7th July 1998 (HPLC fraction 33 – 38 mins) showing the presence of cholest-4-en-3-one (**LVII**). Library mass spectra and co-injection experiments confirm the presence of **LVII** in the SPM.

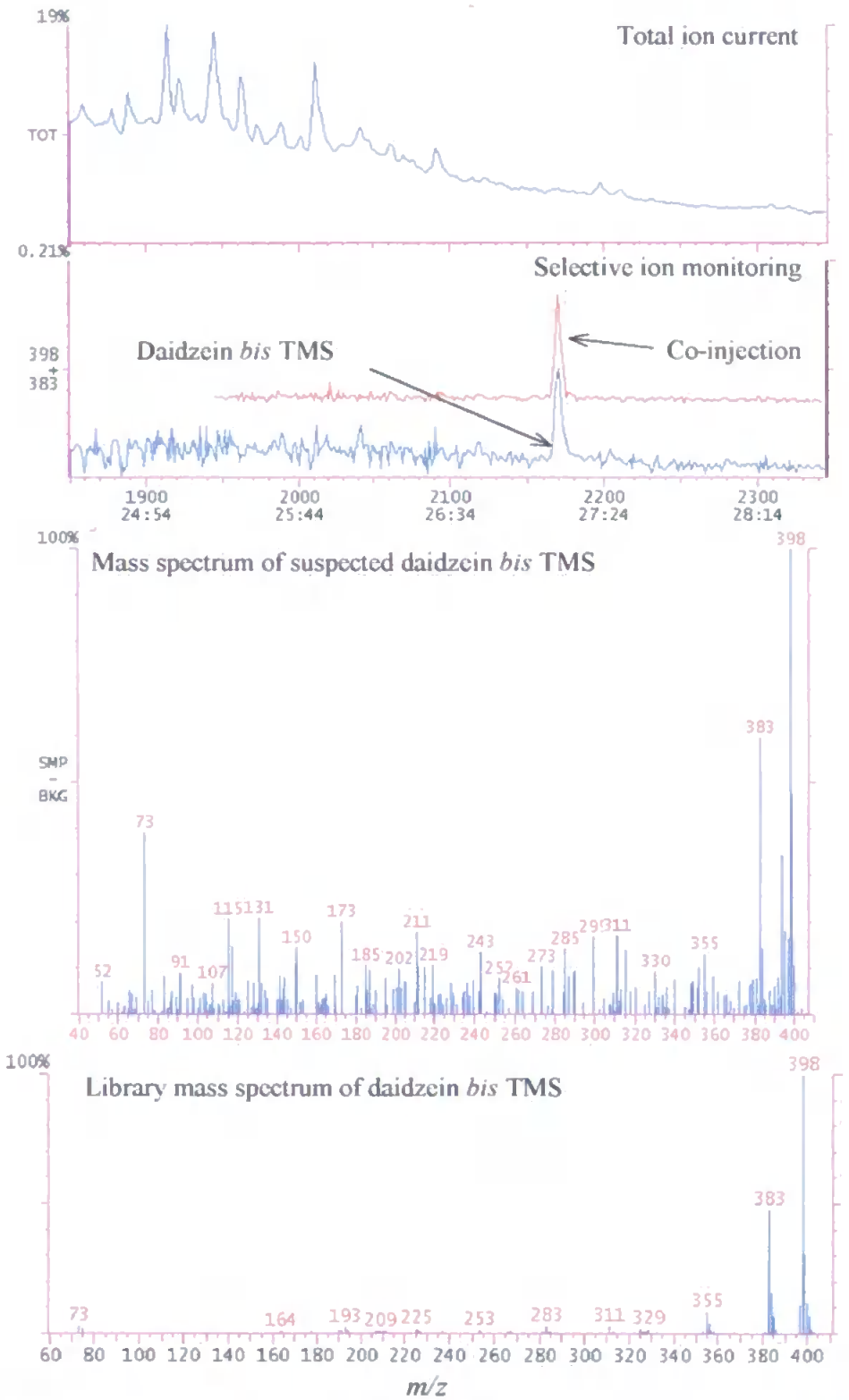


Figure 5.16 GC-MS analysis of Deephams STW effluent taken at 1415 h 7th July 1998 (HPLC fraction 21.5 – 23 min) showing the presence of daidzein (**VIII**) *bis* TMS. Library mass spectra and co-injection experiments confirm the presence of **VII** in the effluent.

Solid samples obtained from Deephams as a result of SPE pre-filter or filtered directly at source through a 10 μm nylon mesh, were all extracted with methanol and DCM and examined directly by GC-MS (as the TMS ether derivatives) or occasionally *via* an initial HPLC cleanup step. The HPLC cleanup procedure used was the same as that used in the effluent fractionation step, prior to GC-MS.

17β-estradiol (**I**), estrone (**IV**) and 16α,17β-estriol (**V**) were not observed in any solid samples examined. The concentrations of natural sterols were examined in all SPE pre-filter samples and thus direct comparison with the appropriate values in the aqueous phase can be made. Such a comparison could not be made for directly taken samples as the aqueous phase was not retained for analysis. However, the concentrations obtained for these solid samples can be attributed to a specific volume of water as this was recorded. The concentrations of solid associated sterols, in most cases, showed a marked increase in concentration compared to levels encountered in the liquid samples. Figure 5.17,*a,b,c,d* shows the concentration of 4 sterols in SPM samples taken directly or as a result of SPE pre-filter, throughout 1998 and a time series. Figure 5.18 shows a typical GC-MS chromatogram for extracted SPM. The concentration of coprostanol (**LXXII**) from SPE inline filter, ranged from 435-1380 ng L^{-1} which is a 4-fold increase in levels determined in the liquid phase.

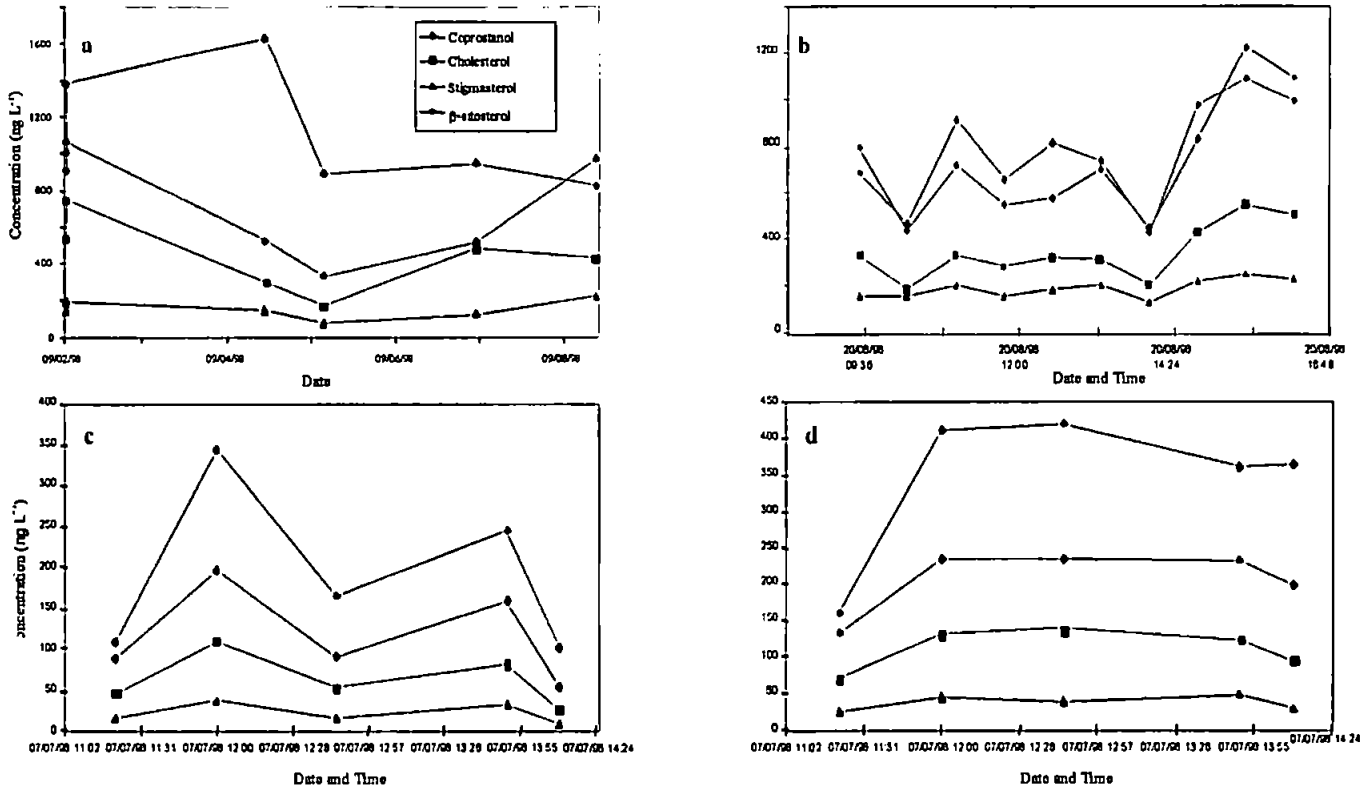


Figure 5.17 Concentration of 4 major sterols, coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**) from SPM from Deephams STW effluent. a/ SPE pre-filter samples taken throughout 1998, b/ SPE pre-filter samples taken from time-series, c/ concentration (ng L^{-1}) of directly taken samples throughout 1998, d/ concentration (ng mg^{-1} DW) directly taken samples throughout 1998.

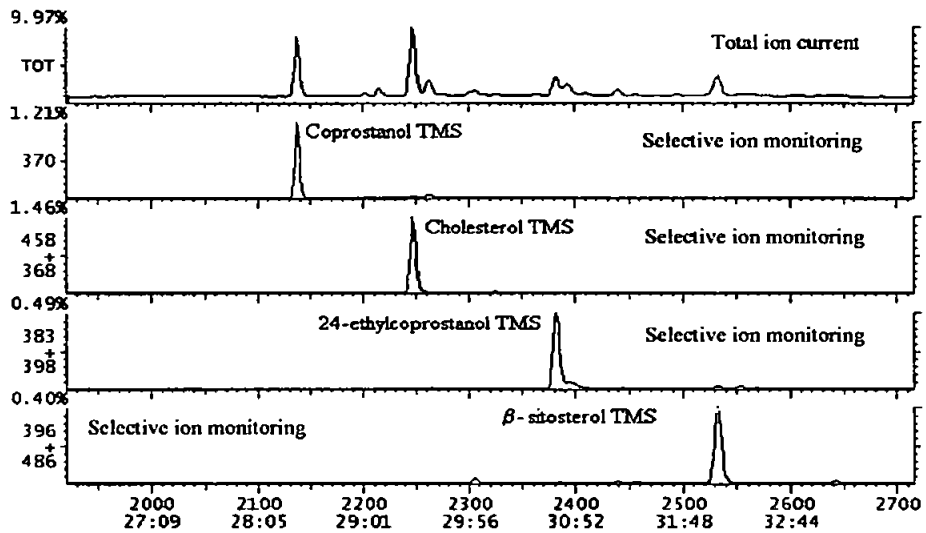


Figure 5.18 Partial GC-MS total ion current and selective ion monitoring chromatograms of four major sterols (as TMS ether derivatives) identified in SPM associated with Deephams STW effluent, taken at 1400 h 22nd April 1998,.

The concentrations measured in directly taken samples was lower, (100-345 ng L^{-1}) possibly due to the loss of fines through the mesh. The amounts per mg dry weight of solids these levels ranged from 159-616 ng mg^{-1}. Cholesterol (**III**) concentrations measured were from 170-745 ng L^{-1} in SPE pre-filter samples and 70-150 ng mg^{-1} in direct samples. The levels of β-sitosterol (**XXXI**) were approximately an order of magnitude greater than amounts seen in the liquid, 332-1093 ng L^{-1} for the SPE filter and 133-234 ng mg^{-1} in direct method (see Appendix A). The concentration of stigmasterol (**LXVI**) in the solid samples ranged between 73-254 ng L^{-1} in the SPE pre-filters and 25-48 ng mg^{-1} in the direct method. NCT (**LXXXI**) was detected in two SPE pre-filter samples taken 9th February and was confirmed by co-injection (Figure 5.19). The concentrations varied considerably between the two samples from 39 ng L^{-1} to 136 ng L^{-1}. NCT (**LXXXI**) was not detected in any other solid sample taken from Deephams STW.

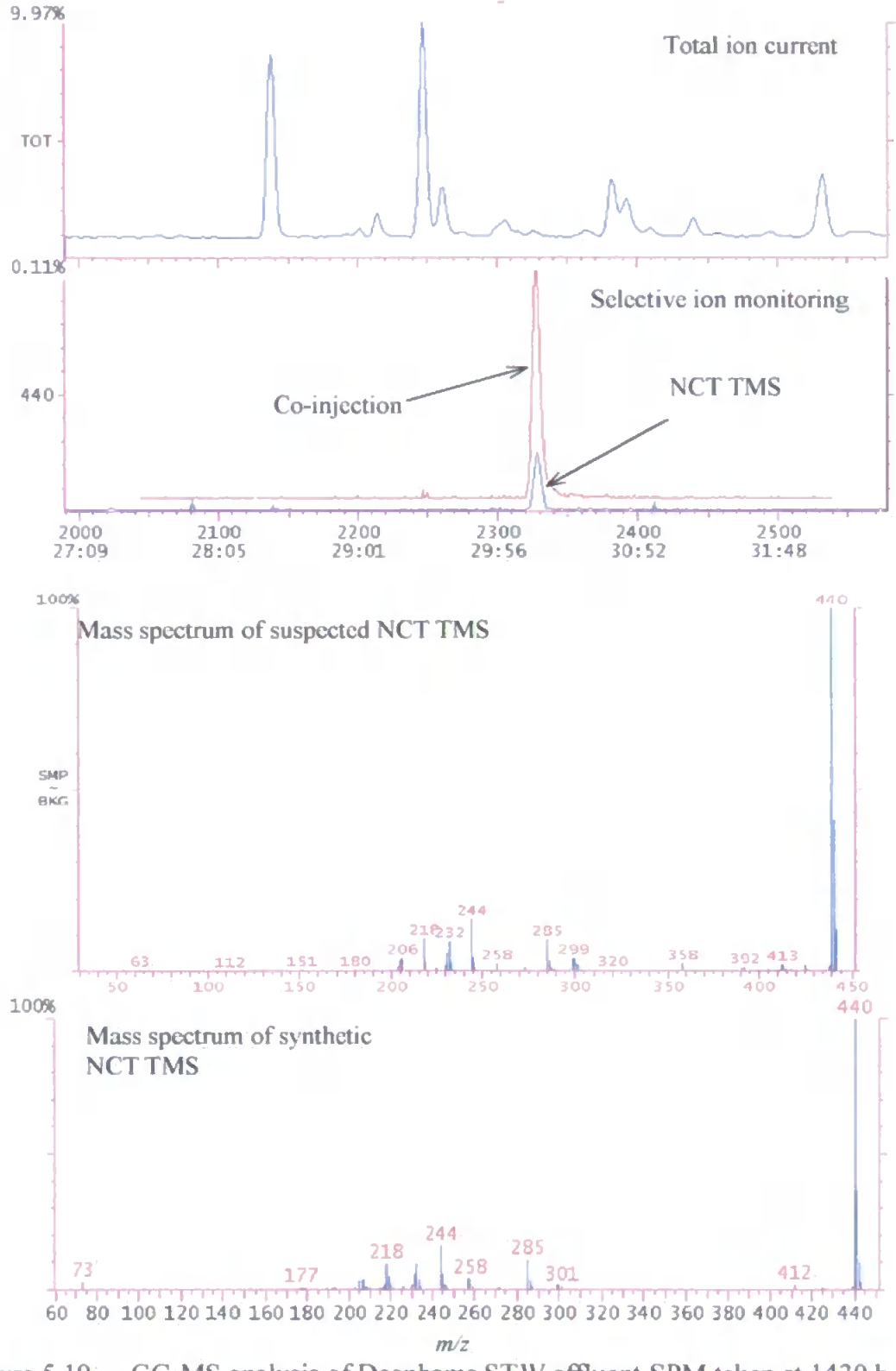


Figure 5.19 GC-MS analysis of Deephams STW effluent SPM taken at 1430 h 9th February 1998 (HPLC fraction 33 – 38 mins) showing the presence of NCT (**LXXXI**) TMS. Library mass spectra and co-injection experiments confirm the presence of **LXXXI** in the SPM.

A summary of estrogens concentration measured from Harpenden STW effluents is shown in Figure 5.20.

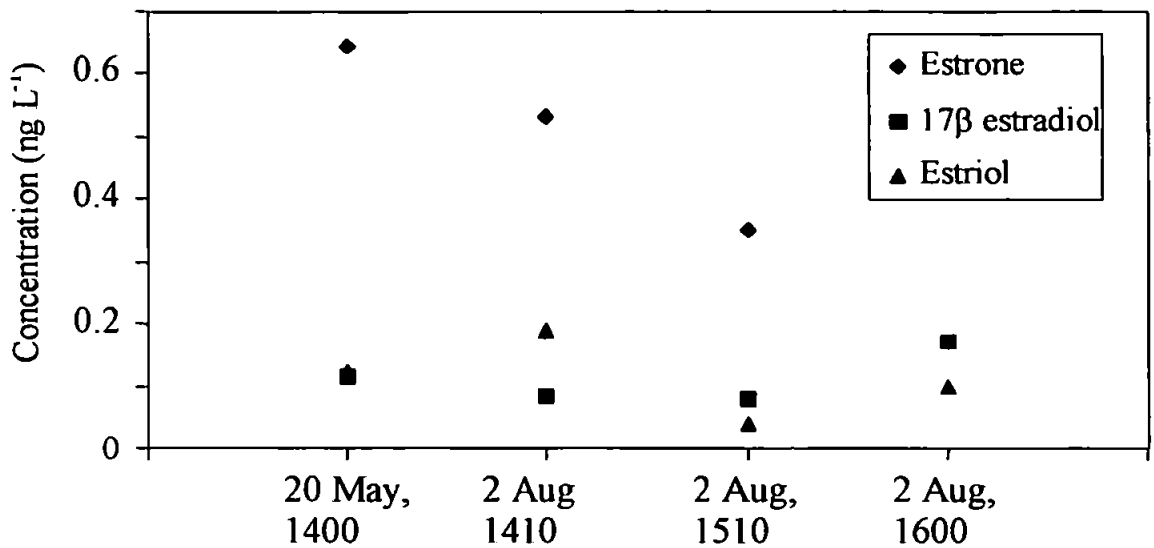


Figure 5.20 Concentrations of estrone (**IV**), 17β-estradiol (**I**) and 16α,17β-estriol (**V**) detected in Harpenden STW effluent over the sampling period.

The concentrations of estrone (**IV**) measured ranged from <LOD to 0.6 ng L^{-1}. The levels of 17β-estradiol (**I**) were lower than for **IV**, ranging between 0.1-0.2 ng L^{-1}. 16α,17β-estriol (**V**), the third most potent natural estrogen, was determined in most effluent samples taken an confirmed by co-injection. Concentrations of **V** ranging from 0.06-0.2 ng L^{-1} (Figure 5.21).

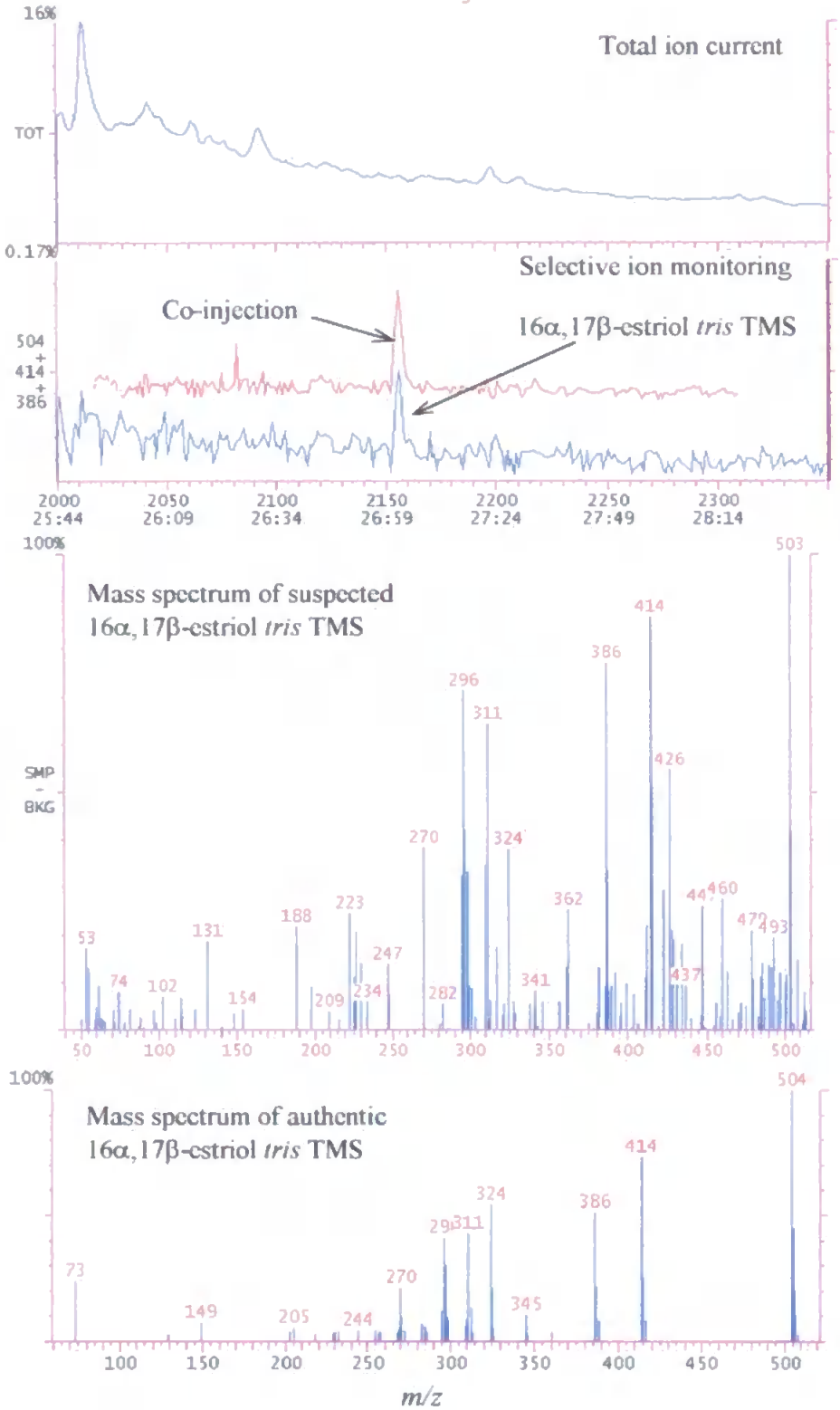


Figure 5.21 GC-MS analysis of Harpenden STW effluent taken at 1410 h 2th August 1998 (HPLC fraction 21.5 – 23 min) showing the presence of 16α,17β-estriol (**V**) *tris* TMS. Library mass spectra and co-injection experiments confirm the presence of **V** in the effluent.

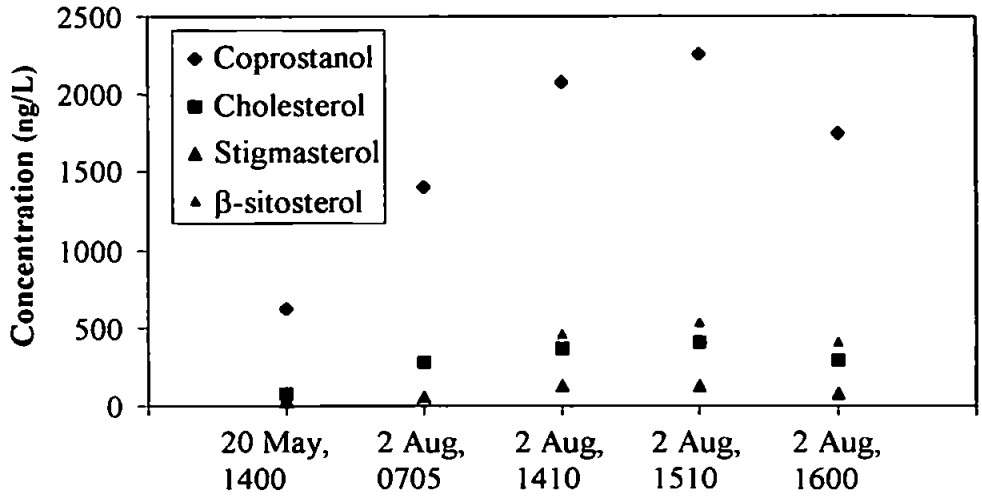


Figure 5.22 Concentrations of coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**) found dissolved in Harpenden STW effluent throughout 1998.

Effluent samples taken at 07:05 2nd August showed that all natural estrogens were <LOD. 17α-ethynylestradiol (**XIII**) and NCT (**LXXXI**) were not detected (<LOD) in any effluent samples. The phytoestrogen, daidzein (**VIII**), was occasionally detected in effluent samples, 1.65 ng L^{-1} being the highest concentration found. No other phytoestrogen was detected. Cholest-4-en-3-one (**LVII**) and cholest-1,4-dien-3-one (**LXIV**) were identified in a number of samples with the maximum concentration being 6.7 ng L^{-1} and 6.5 ng L^{-1} respectively. The major sterols, coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**) were all present in the effluent (Figure 5.22). **LXXII** was identified in the greatest quantity, 625-2261 ng L^{-1}. **III** and **XXXI** were present at comparable concentrations, 82-406 ng L^{-1} and 84-536 ng L^{-1} respectively. **LXVI** concentrations ranged from 20-125 ng L^{-1}.

Solid samples were extracted either from the SPE pre-filter or *via* the direct filter method. In all SPM samples taken from Harpenden STW estrone (**IV**), 17β-estradiol

(**I**), 16α,17β-estriol (**V**), 17α-ethynylestradiol (**XIII**) and NCT (**LXXXI**) were not detected (<LOD). The concentrations of coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**)associated with SPM from SPE pre-filter, are shown in Figure 5.23, and directly taken samples are shown in Figure 5.24, from Harpenden STW effluent.

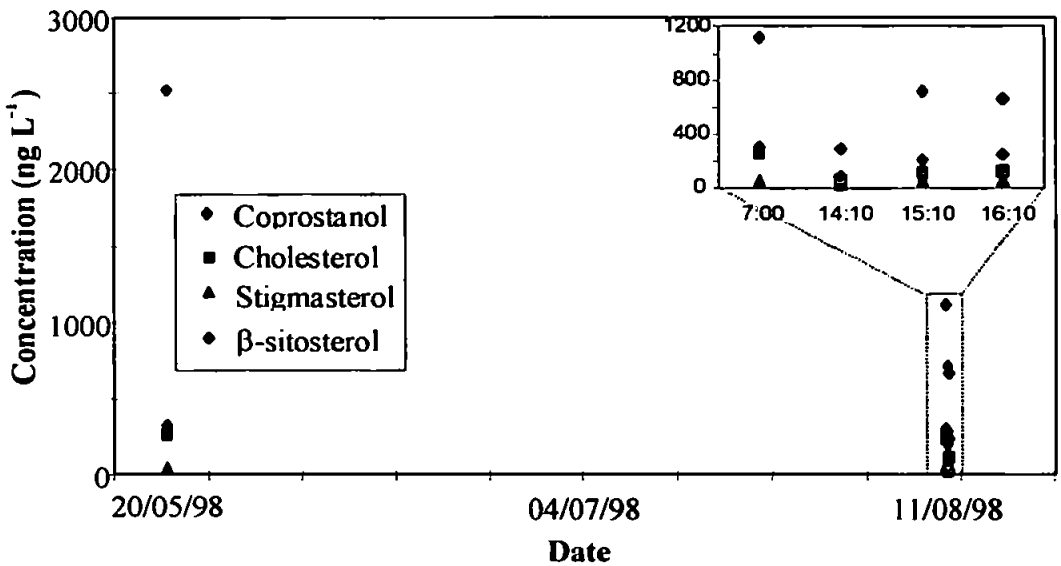


Figure 5.23 Concentrations of coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**) in Harpenden STW effluent SPM SPE filter. Insert shows the variability during a time series in August 1998.

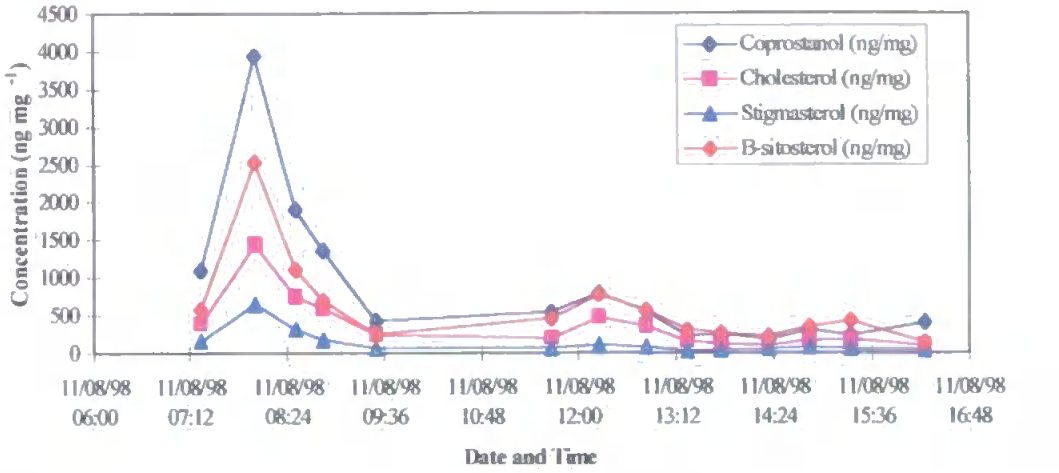


Figure 5.24 Time series concentrations of coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**) in extracted SPM taken directly from Harpenden STW effluent (11th August 1998).

A comparison of highest and lowest concentrations of coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI**) in pre-filter and directly taken SPM samples are shown in Figure 5.25.

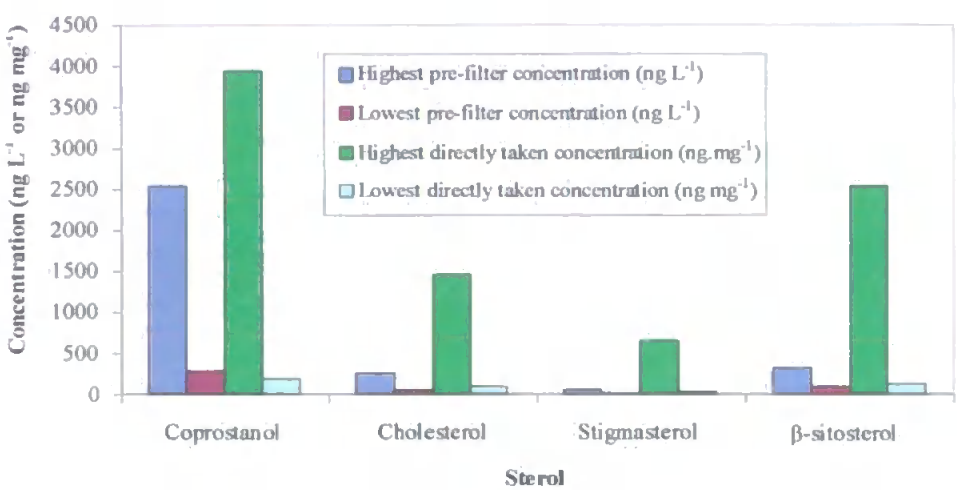


Figure 5.25 Comparison of highest and lowest concentrations of coprostanol (**LXXII**), cholesterol (**III**), stigmasterol (**LXVI**) and β-sitosterol (**XXXI** from pre-filter and directly taken Harpeden STW SPM samples.

5.8 Discussion

On average females excrete 14 μg d^{-1} estrogens in the urine in the conjugated form (Adlercreutz *et al.*, 1986). However, faeces contain up to 2.2 μg d^{-1} estrogens generally in the unconjugated form (Adlercreutz *et al.*, 1986). Thus, on average a total of *ca* 16 μg d^{-1} $female^{-1}$ enters STW. Concerns of the risk to human health of the natural estrogens content of sewage effluents has been recognised for over 25 years. Tabak and Bunch (1970) were the first to examine the metabolism of natural estrogens such as 17β-estradiol (**I**) and synthetic estrogens such as 17α-ethynylestradiol (**XIII**) during sewage treatment. Their results showed that the natural estrogens were less resistant to biologically mediated metabolism than the synthetic estrogens. Further work by Tabak *et al.* (1981) examined the influent and effluent of 14 STWs. The average concentration in influent samples of free (unconjugated) natural estrogens ranged between 10-80 ng L^{-1} whereas the synthetic estrogens were as high as 1.2 μg L^{-1}. The authors examined effluent samples and determined that STWs employing only primary treatment had, at best, a 25 % removal rate of synthetic estrogens (35 - 55 % for natural estrogens). Secondary treated effluents had the synthetic estrogenic signature reduced by a maximum of 40 %, compared with 50 – 70 % for the natural estrogens. There was, therefore, an appreciable amount of **I**, estrone (**IV**) and 16α,17β-estriol (**V**) remaining in the treated effluent, on average 10 ng L^{-1}, 20 ng L^{-1} and 40 ng L^{-1} respectively. The average concentration of **XIII**, however, was 810 ng L^{-1} which would be cause for concern. Further work by Purdom *et al.* (1994) and Harris *et al.* (1996) examining STW effluents and showed that caged male trout placed outside STW outfalls produced the female egg protein, vitellogenin, an estrogenic indicator. This confirmed Tabak *et al.* (1981) results that STW effluents were estrogenic in nature. Recently Desbrow *et al.*

(1998) examined effluents from a suite of STWs in the UK using a similar protocol to that used herein. The author found that the natural estrogens, **I** and **IV** (but not **V**) were present in all effluents at varying concentrations (from 1 ng L^{-1} to 50 ng L^{-1} and 80 ng L^{-1} respectively). The synthetic estrogen, **XIII** was, however, only occasionally found (primarily in STW employing primary treatment). Ternes *et al.* (1999a) examined effluent samples from Canada and Germany, Table 5.8, and found that **I** was only identified in the Canadian effluents. Both **IV** and **XIII** were measured at ng L^{-1} concentrations from both countries.

Estrogen	Canadian effluent (ng L^{-1})	German effluent (ng L^{-1})
Estrone (**IV**)	3	9
17β-estradiol (**I**)	6	<LOD
17α-ethynylestradiol (**XIII**)	9	1

Table 5.8 Average concentrations of estrone (**IV**), 17β-estradiol (**I**) and 17α-ethynylestradiol (**XIII**) found in sewage effluents from Canada and Germany (Ternes *et al.*, 1999a)

Belfroid *et al.* (1999) examined domestic waste water effluent samples from the Netherlands over a 3 month period, Table 5.9. **I**, **IV** and **XIII** were all identified. There was, however, a factor of *ca* 20 difference in concentration between samples taken in October and December from the same site.

Site and Date	Estrone (IV) (ng L^{-1})	17β-estradiol (I) (ng L^{-1})	17α-ethynylestradiol (XIII) (ng L^{-1})
C (October, 1997)	2.1	<0.6	<0.3
C (December, 1997)	47	12	7.5

Table 5.9 Variability of estrogenic compounds taken from the same domestic effluent at different times of the year (Belfroid *et al.*, 1999).

Belfroid *et al.* (1999) also examined industrial effluents for estrogenic potential. On average both **I** and **XIII** were <LOD with only **IV** being identified (average value of 0.4 ng L^{-1}).

The present study examined two STW effluents for estrogenic content, Deephams and Harpenden STWs, over the course of 1998. The concentrations found are shown in Table 5.10.

Site	Date	Estrone (IV) (ng L^{-1})	17β-estradiol (I) (ng L^{-1})	16α,17β-estriol (V) (ng L^{-1})	17α-ethynylestradiol (XIII) (ng L^{-1})
Deephams	9/2/98	3.4#	0.7#	<LOD	<LOD
	22/4/98	0.3#	0.2#		
	13/5/98	0.2#	0.4#		
	7/7/98	3.1#	1.0#		
	20/8/98	0.5#, 0.4*#	0.3#, 0.2*#		
Harpenden	25/5/98	0.6#	0.1#	0.12#	<LOD
	2/8/98	0.44*#	0.11*#	0.11*#	

Table 5.10 Estrogens examined and concentration range determined in effluent samples from Deephams and Harpenden STW over 1998.

*Values averaged over time series.

#Values not corrected for extraction efficiency (*ca* 30 – 50 % extraction efficiency)

Desbrow *et al.* (1998) examined effluents from Deephams and Harpenden on three occasions (17/7/95, 24/7/95 and 1/8/95). The average values measured are shown in Table 5.11.

	Date	IV (ng L^{-1})	I (ng L^{-1})	V (ng L^{-1})	XIII (ng L^{-1})
Deephams	17/7/95 24/7/95 1/8/95	13 2 9.4	12 4.9 4.3	nd	<LOD
Harpenden	17/7/95 24/7/95 1/8/95	5.2 8.5 8.9	3.7 7.1 4.4	nd	<LOD

Table 5.11 The average concentration for natural estrogens detected in effluents from Deephams and Harpenden STWs (Desbrow *et al.*, 1998).
nd, not detected.

Comparing the work of Desbrow *et al.* (1998) for samples taken in 1995 with the present study it is obvious that the uncorrected concentrations in the Desbrow study were generally higher. The estrogen, 16α,17β-estriol (**V**), was identified in effluents from Harpenden in the present study but not by Desbrow and co-workers. 17α-ethynylestradiol (**XIII**) was <LOD in both studies. **XIII** is efficiently retained by the SPE cartridge (~79 %, 2 SPE method, Desbrow *et al.*, 1998) and elutes in the same HPLC fractions as estrone (**IV**) and 17β-estradiol (**I**). Comparing the data in Tables 5.10 and 5.11 the difference in concentration is partly due to the greater extraction efficiency, 83.3 % - 85.4 % for the twin SPE method (53.4 % -53.8 % herein). **V** was not measured by Desbrow and co-workers but was found to have extraction efficiency of 31.1 % herein (see Table 5.4, page 154). The difference in performance of the two methods is *ca* 2 which is, however, insufficient to account for a maximum difference of *ca* 35 when comparing maximum **I** concentrations from Harpenden STW. The

minimum difference between the data sets is *ca* 4 comparing **IV** concentrations from Deephams STW. There are other physical changes that could account for the lower levels determined herein in 1998. Changes in operational practice at the two sites may have occurred following the publication of the Desbrow finds though non-has been reported. Dilution effects due to differences in rainfall may have also influenced the concentrations.

The difference in values between Desbrow and co-workers and the present study could also in part be attributed to the time of day at which samples were taken. Samples in the present study were generally taken at *ca* 1400 h (Table 5.1). Desbrow *et al.* (1998) do not report the times at which their samples were taken. Figure 5.11 shows the variability of 17β-estradiol (**I**) and estrone (**IV**) in Deephams effluent over a 7 h time series. The **I** concentration shows a variability factor of *ca* 5 whereas **IV** was *ca* 12. In order to obtain the maximum values in the effluent (*i.e.* during times of heavy input such as late morning (Tchobanoglous and Burton, 1991)) the specific residence time of the plant should be determined. Therefore, the time of sampling and residence time in the plant are important variables. Belfroid *et al.* (1999) found a factor of *ca* 20 difference in **IV**, **I** and 17α-ethynylestradiol (**XIII**) concentrations between samples taken in October and December from the same sampling site in the Netherlands. The author reports that the difference was due to the concentration in the influent. Such a difference in influent concentrations could be the result of storm water causing rapid dilution. During storm weather, the STW is unable to cope with the increased volume of influent. A percentage of influent is lost to storm channels (*i.e.* bypasses the STW) resulting in lower estrogen levels entering the STWs.

The lower estrone (**IV**) concentration reported in the present study can be accounted for by lower extraction efficiencies, possible rainwater dilution and the times at which samples were taken but these variables do not fully account for the much lower values of 17β-estradiol (**I**) encountered. One further possible factor governing the elevated **I** levels reported by Desbrow and co-workers is due to the analytical method used. Desbrow and co-workers added the external standard, 1,4-^{2}H-**I** to all HPLC fractions prior to GC-MS monitoring with *m/z* 272 ($\mathbf{M}^{+\cdot}$) and 213. Evidence herein suggests that these ions are also present in the mass spectrum of 1,4-^{2}H-**I** (Figure 5.26).

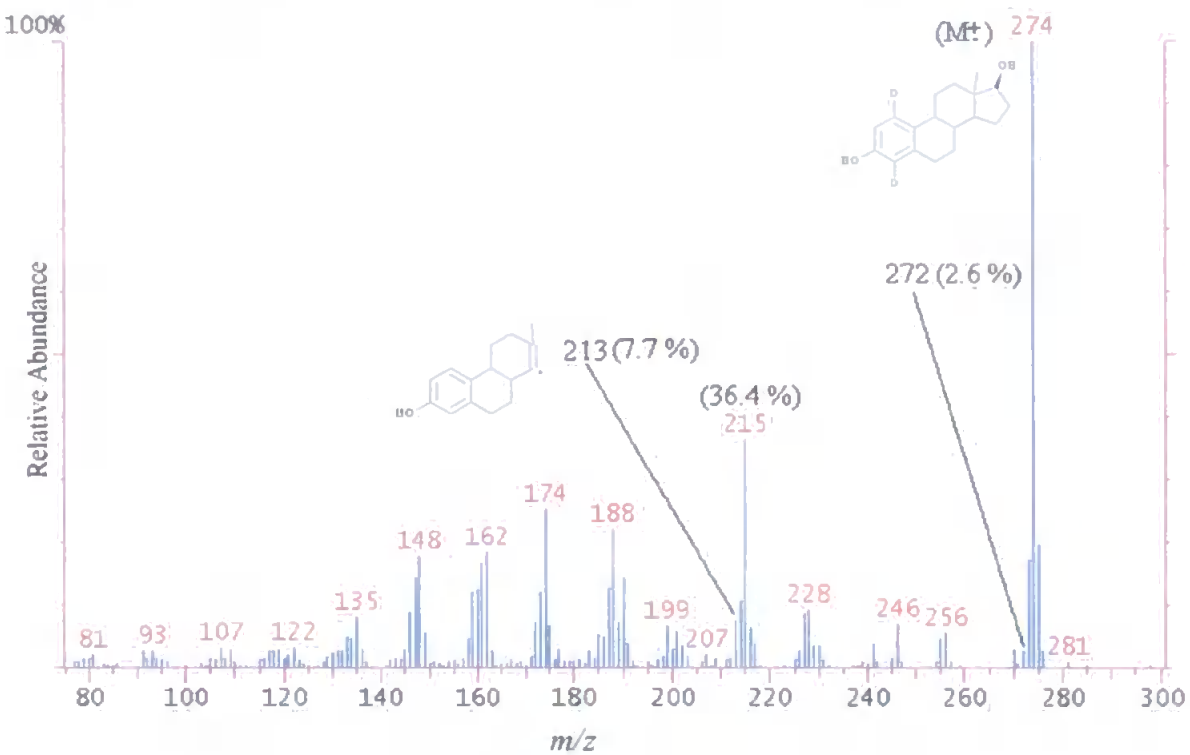


Figure 5.26 Mass spectrum of 1,4-^{2}H-17β-estradiol (**I**) showing the relative abundances of *m/z* 272, 215, 213 and 274 ($M^{+\cdot}$).

Since 17β-estradiol (**I**) and 1,4-^{2}H-**I** co-eluted on a HP5-MS stationary phase a correction needs to be made for 1,4-^{2}H-**I**. No such correction was reported by Desbrow *et al.* (1998) and the analytical method has been subsequently refined whereby the mono *tert*-butyldimethylsilyl ether derivative is prepared (Kelly, 2000).

16α,17β-estriol (**V**), a metabolite of 17β-estradiol (**I**), is the third most potent natural estrogen. Although females excrete **V** naturally as a result of menstruation, concentrations in the urine are dramatically increased during pregnancy compared with **IV** and **I** (Table 5.13).

	V in urine (mg d^{-1})	**IV** in urine (mg d^{-1})	**I** in urine (mg d^{-1})
Male	0-0.01*		
Non pregnant female, averaged over 28 day cycle	0-0.06*		
27 weeks gestation	6.2	1.2	0.4
30 week gestation	5.5	1.2	0.9
36 week gestation	20.3	1.7	0.5

Table 5.13 Comparison of estrone (**IV**), 17β-estradiol (**I**) and 16α,17β-estriol (**V**) concentrations in urine during the menstrual cycle and pregnancy (Lamparczyk *et al.*, 1994).

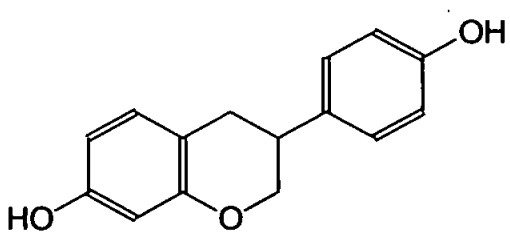
* data taken from http://anatomy.adam.com/peds/top/003620.htm .

Reports of the identification of 16α,17β-estriol (**V**) being identified in sewage effluent are limited (Tabak *et al.*, 1981; Lee and Peart, 1998; and herein). Tabak *et al.* (1981) found that the average **V** levels in influent samples was 80 ng L^{-1} compared with, on average, 40 ng L^{-1} in effluent samples. Lee and Peart (1998) examined 5 Canadian STW for estrogenic content (estrone (**IV**), 17β-estradiol (**I**) and **V**). The **V** concentration in the influent was as high as 250 ng L^{-1} which was much greater than the **IV** and **I** concentration determined (41 ng L^{-1} and 15 ng L^{-1} respectively). The

concentration in the effluent was reduced to 30 ng L^{-1} for 16α,17β-estriol (**V**) a removal rate of 88 % compared with 14 ng L^{-1} and <LOD for estrone (**IV**) and 17β-estradiol (**I**). In the present study **V** was identified in all samples taken from Harpenden STW (<LOD in effluent samples taken from Deephams) albeit at sub ng L^{-1} levels (Table 5.10). Correcting for the SPE extraction efficiency (efficiency was just 31 % for **V**) the true **V** concentration in the effluent may have been greater than that of **I** (~0.3 ng L^{-1} **V** compared with ~0.2 ng L^{-1} **I**). The **V**:**I** ratio in the effluent was 1.5:1. **V** was not detected by Desbrow *et al.* (1998) due to the analytical protocol used. The average concentration of **I** determined in the Harpenden effluent by Desbrow and co-workers, was 5.1 ng L^{-1}. Applying the ratio it is hypothesised that the average concentration of **V** in the effluent would be *ca* 7.5 ng L^{-1}. Routledge and Sumpter (1997) reported that 16α,17β-estriol (**V**) was *ca* a factor of 300 fold less active than 17β-estradiol (**I**) when tested *in vitro*. Thus a concentration of 7.5 ng L^{-1} would have the equivalent estrogenic activity as 0.025 ng L^{-1} **I**. The limit of detection of **I** by the Yeast Assay is *ca* 2 ng L^{-1} (Routledge and Sumpter, 1996) and hence it is unlikely that such a level of **V**, theoretically present in their effluent samples, would have given a response above background levels. Finally, the HPLC fractions analysed by GC-MS would not have contained **V** due to the fact that it has a much shorter retention time (~3 minutes) compared with **IV** and **I** under the conditions used.

The isoflavone phytoestrogen, daidzein (**VIII**), has been found to be present in tofu, soy based products as well as popular beverages such as beer (Rosenblum *et al.*, 1992; Dwyer *et al.*, 1994; Reinli and Block, 1996; Bingham *et al.*, 1998). **VIII** has not, however, been identified in sewage effluent prior to this study. The concentrations of **VIII** in human urine can be >100 μg L^{-1} (Bannwart, 1984; Lu *et al.*, 2000). Although

there is currently no data on **VIII** metabolism during wastewater treatment it is known that **VIII** is easily metabolised by the mammalian gut bacteria to produce equal (**LXXXXVIII**) (Hodgson *et al.*, 1996; King 1998). In women **VIII** intake suppresses estrone (**IV**) biosynthesis by producing 2-hydroxyestrone and thereby lowering 17β-estradiol concentration (Lu *et al.*, 2000). The closely related phytoestrogen genistein (**IX**), also present in soya products at high concentrations, undergoes rapid hydroxylation and methylation of the two aromatic rings by *Streptumyces griseus* and *Streptomyces catenulae* bacteria. (Hosny and Rosazza, 1999). No metabolites were determined for **VIII**.



Equal (**LXXXXVIII**)

The induction of vitellogenin in Siberian sturgeon (*Acipenser baeri*) exposed to **VIII** shows it has an estrogenic potential, albeit at levels far lower than 17β-estradiol (**I**) (**VIII** is *ca* 250 000 fold less active an estrogen than **I**) (Pelissero *et al.*, 1991). **VIII** was identified by full scan GC-MS, periodically in Deephams and Harpenden effluent samples in the range of 1.65-3 ng L^{-1}. At such levels the yeast assay would not be sensitive enough since 3 ng L^{-1} **VIII** would have same estrogenic potential as 0.012 pg L^{-1} **I**, >5 orders of magnitude below the LOD. Thus, the concentrations of **VIII** found herein contribute little to the overall estrogenicity of the STW effluent.

Solids associated with STW such as activated sludge/sewage sludge have been examined previously for the specific organic signature such as specific sterols and fatty acids (McCalley *et al.*, 1981; Quéméneur and Marty, 1994; Nguyen *et al.*, 1995). Bile acids, excreted from the body at high concentrations, have also been analysed in influent and effluent samples (liquid and solid) and have been successfully used in determination of sewage pollution in sediments (Elhmmali *et al.*, 1997; Elhmmali *et al.*, 2000). The xenoestrogen 4-nonylphenol (**XVIII**) has been identified in sewage sludge (Smoot and Taylor, 1984; Ejlertsson *et al.*, 1999; Sekela *et al.*, 1999) but to date no steroidal based compound with an estrogenic potential has been reported to be associated with solid matter. Desbrow *et al.* (1998), during the course of SPE of effluent samples, removed solids *via* the pre-filter which were extracted. The extracts were fractionated by HPLC and these tested by the YES assay. No fractions gave an estrogenic response above background. Quéméneur and Marty (1994) examined the sterol content of aqueous and SPM in wastewater effluents and found that 94 % of cholesterol (**III**), was associated with the SPM.

For all SPM samples analysed the steroidal estrogens, estrone (**IV**), 17β-estradiol (**I**), 16α,17β-estriol (**V**) and 17α-ethynylestradiol (**XIII**) were not detected. Estrogens entering the STW in the influent are generally in the conjugated state and dissolved. However, ~14 % in the unconjugated state is associated with solids (Adlercreutz *et al.*, 1986). Ternes *et al.* (1999b) studied the metabolism of 17β-estradiol glucuronide, 17β-estradiol-17(β-D- glucuronide) and 17β-estradiol-3(β-D- glucuronide) during sewage treatment and showed that within 15 minutes **I** and **IV** were found at detectable levels. **IV** concentrations reached a maximum level after 20 h and then dropping off to <LOD after 60 h. In a similar experiment Ternes *et al.* (1999b)

showed that the metabolism of **I** was rapid with 95 % being oxidised to **IV** in 3 h. After ~ 5 h **I** was below the limit of detection (Ternes *et al.*, 1999b). These results show that the conjugated estrogens have a limited degree of protection from metabolism *via* the inclusion glucuronide or sulphate conjugates. The 'free' estrogens, (in solid material) coming from a smaller concentration pool are rapidly metabolised and hence not identified in SPM samples. **IV** and **I** seen in the aqueous effluent fractions therefore are most likely to have originated from the conjugated estrogens.

In Chapter 1 it was hypothesised that cholesterol (**III**) in STW, might aromatise to A-ring aromatic steroids such as NCT (**LXXXI**). If **LXXXI** were present in the effluent this would add to the overall estrogenicity. Aromatisation of **III**, in mammalian systems, to form natural estrogenic compounds is P_{450} mediated (Devlin, 1997) and hence if organisms present in sewage also possess the P_{450} cytochrome then A-ring aromatisation of **III** is a possibility. In addition to **LXXXI** exhorting estrogenicity it has also been shown to degrade by some bacteria to estrone (**IV**) (Afonso *et al.*, 1966). Thus it was important as a complement to the study of radiolabelled cholesterol (**III**) incubation in STW influents (Chapter 4) to monitor real STW for the presence of NCT (**LXXXI**).

Previous studies of NCT (**LXXXI**) have been confined to chemical synthesis in the laboratory (Afonso *et al.*, 1966; Sengupta *et al.*, 1987; Suginome *et al.*, 1988; Kocovský and Baines, 1994). There has been no examination of the possible biosynthesis of **LXXXI** in the environment. Since **LXXXI** has not been shown to be produce from cholesterol (**III**) herein the **LXXXI** identified can either be attributed to the metabolism of an, as yet, unknown compound *in situ* or to a direct contribution *via*

the influent. Furthermore, **LXXXI** in the treatment liquor could either bioaccumulate or be a transient intermediate. The results in Chapter 4 failed to establish that the metabolism of cholesterol (**III**) produced **LXXXI**. For **III** to be converted to **LXXXI** requires rearrangement, oxidation, dehydration and aromatisation to occur. From the results of STW monitoring **LXXXI** is a minor, intermittent component of STW effluent which in turn could be a reflection on the relatively low abundance and intermittent nature of the reactant.

The major route of bacterial steroid metabolism initially occurs *via* β-oxidation of the alkyl side chain (extensively reviewed in Chapter 1). Data reported herein showed that *ca* 25 % of radiolabelled cholesterol (26-^{14}C-**III**) underwent β-oxidation of the alkyl side chain, when incubated in a laboratory STW, resulting in mineralisation to $^{14}CO_2$ in the first 24 h of incubation (see Results, Chapter 4). **LXXXI** either entering the STW in the influent or being produced *in situ* during sewage treatment would be subjected to the same oxidation processes and thus may be a transient intermediate. Afonso *et al.* (1966) showed that NCT (**LXXXI**) was metabolised by the bacterium *Nocardia restrictus* to form estrone (**IV**) (8 %) over 240 h. No data exists on the rate of **LXXXI** metabolism during sewage treatment. If **LXXXI** is indeed a transient intermediate a possible end product could be **IV**.

Given the high computed log K_{ow} value for **LXXXI** (Table 5.2) it was to be expected that any significant **LXXXI** burden would be associated with SPM rather than aqueous samples. Indeed this proved to be the case but **LXXXI** was identified in only two STW SPM samples taken from Deephams STW on the same day. **LXXXI** was not identified at all in any of the Harpenden SPM samples. The concentration of NCT

(**LXXXI**) found on the SPE pre-filter was 39 and 136 ng L^{-1} (20 and 68 ng mg^{-1}, taking average SPM as 2 mg L^{-1}). The theoretical log K_{ow} value calculated for **LXXXI** was 9.23 (K_{ow} = 1.7 x 10^9). This is lower, but similar to the value determined for stigmasterol (**LXVI**) (log K_{ow} = 9.43, K_{ow} = 2.7 x 10^9). Since the concentrations of **LXVI** found in solid samples from Deephams were 4 times higher than those dissolved in the effluent it might be reasonable to expect **LXXXI** levels in the aqueous effluent to be about 10-35 ng L^{-1} range if the distribution is at equilibrium. Such levels are well above the LOD but **LXXXI** was not detected in any of the aqueous samples. It is likely that the partitioning between water and SPM is dominated by features of the OM of the particles (K_{oc}) which may be very different than K_{ow} (G. Pan, *personal communications*).

The sampling and analysis of STW influents was not conducted in the present study, however, there have been no published reports of **LXXXI** being identified in STW influent as the free alcohol or as a conjugated steroid (McCalley *et al.*, 1981; Quéméneur and Marty, 1994; Elmmali *et al.*, 1997). The identification of **LXXXI** in STW influents could have been overlooked because the methodology employed previously may not have been optimised for the analysis of such compounds.

Coprostanol (**LXXII**) the mammalian metabolite of cholesterol (**III**), is the most abundant sterol found in human faeces (Lemming *et al.*, 1996) and hence the most abundant sterol found in sewage sludge (Nguyen *et al.*, 1995) and aqueous effluents (Quéméneur and Marty, 1994). **LXXII** is an important indicator of sewage pollution in the environment (McCalley *et al.*, 1981; Leeming *et al.*, 1996; Writer *et al.*, 1995; Mudge and Bebianno, 1997). The higher plant sterol, β-sitosterol (**XXXI**), has been

implicated in causing endocrine disruption to goldfish (MacLathchy and Van der Kraak, 1995). The HPLC fractionation of extracted aqueous and SPM effluent samples used in the investigation of NCT (**LXXXI**) resulted in the natural sterols such as cholesterol (**III**), coprostanol (**LXXII**) and β-sitosterol (**XXXI**) being monitored (majority of sterols and **LXXXI** all elute in 33-40 minute fraction, Table 5.5). The maximum values obtained in samples analysed and the partitioning between liquid and solid are highlighted in Figure 5.27.

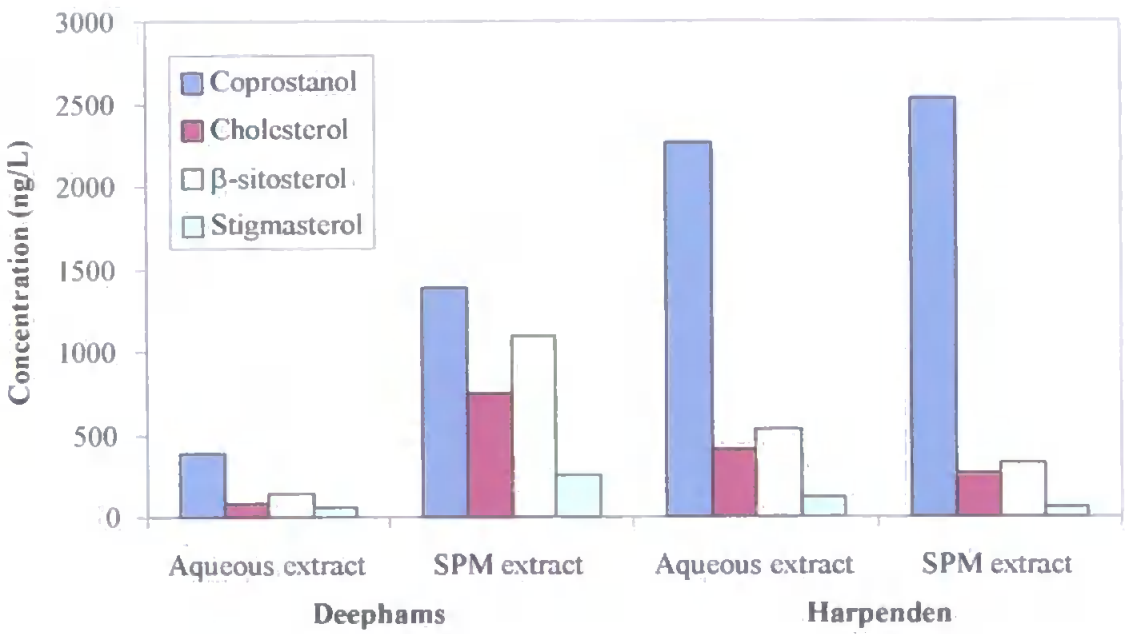


Figure 5.27 Comparison of four natural sterols concentrations found in aqueous and SPM samples monitored from both Deephams and Harpenden STWs in 1998.

Examining the maximum concentrations of the four main sterols present at both Deephams and Harpenden show that the sterol concentrations at Deephams were between 4-9 fold higher in solid samples than in corresponding liquid samples. The ratio of sterols found in the SPM to aqueous phases of the Harpenden STW was 0.5-1.1, which suggests that more sterols were dissolved in the Harpenden effluent than Deephams. The difference between sites is quite dramatic but is most likely due to

fine particulate material passing through the 1.2 μm filter and collecting on the top of the SPE cartridge. Hence, the concentration measured in the aqueous fraction could have an appreciable amount contributed from fine SPM. The examination of fresh effluent samples from Deephams showed that large clumps of solid flocculated in the collecting vessel. The solids associated with the Harpenden effluent were visually finer and tended to remain high in suspension. Quéméneur and Marty (1994) examined an activated sludge STW in Morlaix, France and identified that 2.2 μg L^{-1} cholesterol (**III**), 500 ng L^{-1} coprostanol (**LXXII**), 1.1 μg L^{-1} β-sitosterol (**XXXI**) and 500 ng L^{-1} stigmasterol (**LXVI**) were present in the aqueous effluent (Table 5.14). Such values are all typically higher than those encountered at Deephams and Harpenden. The sterol content of the solids associated with the effluent were generally much higher than those reported here with **III** concentrations 47 times greater and **XXXI**, **LXXII** and **LXVI** an order of magnitude greater (Table 5.14). McCalley *et al.* (1981) analysed effluents from an activated sludge STW in Avonmouth for **III** and **LXXII** levels and found that dissolved concentrations were 600 ng L^{-1} and 400 ng L^{-1} respectively which are in good agreement with the current study (Table 5.14).

Authors	Present study, Deephams		Present study, Harpenden		Quéméneur and Marty, (1994)		McCalley *et al.*, (1981)		Nguyen *et al.*, (1995)	
Sterol	Liquid ($ng\ L^{-1}$)	Solid ($ng\ L^{-1}$)	Liquid ($ng\ L^{-1}$)	Solid ($ng\ L^{-1}$)	Liquid ($ng\ L^{-1}$)	Solid ($ng\ L^{-1}$)	Liquid ($ng\ L^{-1}$)	Solid ($ng\ L^{-1}$)	Liquid ($ng\ L^{-1}$)	Solid ($ng\ mg^{-1}$)
Coprostanol (**LXXII**)	383	1380	2261	2530	500	31 200	400	8 800		4100
Cholesterol (**III**)	80	745	406	264	2 200	32 900	600	12 000		450
β-sitosterol (**XXXI**)	150	1093	536	330	1 100	12 300				280
Stigmasterol (**LXVI**)	66	254	125	60	500	3 600				60

Table 5.14 Comparison of sterols in sewage effluent liquids and solids from the present study (Deephams and Harpenden STWs) and a number of other published works.

Bound to SPM the levels were 12 000 ng L^{-1} and 8 800 ng L^{-1} respectively which converts to 1714 ng and 1257ng per mg of solid. This is *ca* 2 fold greater than coprostanol (**LXXII**) concentrations and an order of magnitude greater than cholesterol (**III**) concentrations determined herein. Nguyen *et al.* (1995) examined sewage sludge for the sterol content (Table 5.14) and found that levels were in fairly good agreement with effluent SPM from Deephams.

The greatest total concentrations (SPM + aqueous extracts) of β-sitosterol (**XXXI**) were 1243 ng L^{-1} and 866 ng L^{-1} for Deephams and Harpenden respectively (Figure 5.27). **XXXI** has been implicated in causing masculinisation in female goldfish (MacLatchy and Van der Kraak, 1995) by lower *in situ* 17β-estradiol (**I**) concentrations. The **XXXI** also reduced plasma testosterone (**XVI**) and 11-ketotestosterone (**XXXII**) in male goldfish. The levels injected into the goldfish were high, 20 and 100 μg g^{-1} (wet weight). Such levels of **XXXI** in the environment are encountered in paper mill effluents, 280 μg L^{-1} in secondary treated effluent (MacLatchy and Van der Kraak, 1995). The greatest concentration of β-sitosterol (**XXXI**) determined herein was ~1 μg L^{-1} (in most samples taken **XXXI** was the second highest sterol identified, coprostanol (**LXXII**) was the highest), which is < 2 orders of magnitude values encountered outside of paper mills. For this study it seems reasonable to assume that although the concentration of **XXXI** in the sewage effluent was high, with respect to other sterols present, it seems unlikely that such levels would cause a disrupting effect on resident fish species. Such levels are constantly being ejected into the environment and hence **XXXI**, if fish bioaccumulate it, could have a disrupting effect sexually. Research is needed in this area to assess the possible implications on native fish species.

The total sterol burdens released into the river Lea are different between the Deephams and Harpenden sites. These differences must be due to the type of treatment employed by the two sites. Deephams uses an activated sludge method while Harpenden employs percolating filters and sand filters. Physically the Harpenden STW has a higher efficiency for removing solids from the liquid (visual observation) possible due to the filtering technique used. The Deephams STW uses secondary activated sludge aeration with a final holding lagoon to remove residual solids. Upon examination of the holding lagoons there was visibly a high solid content released into the effluent channel. McCalley *et al.* (1981) also reported that dissolved coprostanol (**LXXII**) and cholesterol (**III**) in effluents from a percolating filter plant were much higher than those identified in effluents from an activated sludge STW.

In summary the major finding of the monitoring of STW effluent are shown in Table 5.15.

Compound monitored	Concentration range (ng L^{-1})	Limit of detection (ng L^{-1})	Limitation of analysis	Note
NCT	39 – 136	0.1	High log K_{ow} value means problem with extraction.	Found in SPM from Deephams STW effluent only. Not previously identified in the environment.
Estrone	0.1 – 3.4	0.1	Low SPE extraction efficiency.	Consistently most concentrated estrogen found in effluent samples.
17β-estradiol*	0.1 – 1	0.1	Low SPE extraction efficiency.	Much lower concentration than previously found in same STW effluents.
16α,17β-estriol	0.05 – 0.2	0.05	Very low SPE extraction efficiency.	Third most potent natural estrogen rarely found in STW effluents.
17α-ethynylestradiol	< LOD	0.1	Low SPE extraction efficiency.	Greater volume of effluent required for detection.
Daidzein	0.6 – 2.7	0.5	Extraction method not optimised for daidzein.	Not previously identified in STW effluent samples.
Cholesterol	10 - 745	1		
Coprostanol	49 – 2530	1		Consistently most concentrated sterol found in effluent samples.
β-sitosterol	12 - 1065	1		Concentration consistently greater than cholesterol

Table 5.15 Summary of major findings of estrogens in Deephams and Harpenden STW effluents. The concentration of individual compounds determined by response factors using the external standard.

* 17β-estradiol concentrations determined by linear regression and not by response factor with external standard.

5.9 Conclusions

17β-estradiol and estrone were measured in effluents from two STWs near London at concentrations slightly lower but in the same broad range as those reported from these effluents in another recent study (Desbrow *et al.*, 1998). The differences between these 2 studies could be attributed to changes in operational characteristics of the STW between 1995 and 1998 and/or to the variable dilution effects of rainfall, the time at which effluent samples were taken and to small differences in the analytical methods. 16α,17β-estriol was present consistently in the STW effluent from Harpenden (0.05-0.2 ng L^{-1}) effluents but was < LOD in effluents from Deephams STW. Cholest-4-en-3-one was found occasionally in effluents from Deephams and Harpenden, cholest-1,4-dien-3-one was identified in one sample taken from Harpenden STW. Both cholest-4-en-3-one and cholest-1,4-dien-3-one are bacterial

metabolites of cholesterol. A putative A-ring aromatisation product of cholesterol, NCT, was not identified in aqueous samples from Deephams and Harpenden or in solid samples from Harpenden but was seen in two SPM samples from Deephams at concentrations of 39 and 136 ng L^{-1}. The origins of the NCT found in the effluents samples of Deephams STW are as yet undetermined. The phytoestrogen, daidzein which has not previously been found in STW effluents, was identified in Deephams and Harpenden effluents at a maximum concentration of 2.73 and 1.65 ng L^{-1} respectively. The daidzein identified is believed to originate from the natural influent burden. The synthetic estrogen, 17α-ethynylestradiol, was not detected in either liquid or solid samples. The sterols, coprostanol, cholesterol, β-sitosterol and stigmasterol were found in all liquid and solid samples. Coprostanol, a known mammalian metabolite of cholesterol was the most abundant sterol measured and values were comparable to previously reported data for U.K STW. The plant sterol, β-sitosterol was found in all samples, generally at greater concentrations than cholesterol.

Chapter 6

6.1 Overall discussion and conclusions

The primary aim of the present study was to examine whether, under wastewater treatment conditions, cholesterol could undergo A-ring aromatisation to form estrogenic compounds such as NCT, estrone and 17β-estradiol and thus add to the burden of estrogenic steroids in STW effluents. In order to examine this hypothesis three experimental areas were identified. The first involved the synthesis and subsequent chromatographic and spectral characterisation of NCT. Secondly, the metabolism of cholesterol by STW bacteria under laboratory scale simulated sewage treatment conditions was investigated using two ^{14}C radiolabelled cholesterols and finally, monitoring of STW effluents for a range of A-ring aromatic steroids including NCT, estrone, 17β-estradiol and estriol was undertaken. Synthetic NCT was used as a reference compound for positive identification of naturally-occurring NCT

NCT was synthesised from cholest-1,4-dien-3-one by the method of Afonso *et al.* (1966). The synthesis, yielded a total *ca* 50 mg of which 40 mg was ca 85 % pure, used for NMR analysis. The remaining 10 mg was determined to >98 % pure (by GC-MS) and was used as a standard for chromatographic purposes and for estrogenic testing. Few previous studies have produced material pure enough for bioassays, which has been achieved herein and which was a key goal of the synthesis. The newly established yeast assay for determining the estrogenic showed that NCT was estrogenic but *ca* 200 000 fold less active than 17β-estradiol at the minimum effect. The log K_{ow} was calculated and found to be 9.23. The high log K_{ow} casts some doubt over the reliability of the yeast assay since such hydrophobic compounds may be relatively insoluble in the medium used for the assay. Other assays such as

determination of *in vivo* production of vitellogenin in trout should probably be performed with NCT due to the fact that metabolism produces estrone.

The study of cholesterol metabolism during sewage treatment involved the use of both radiolabelled C-4 and C-26 cholesterol in standard laboratory-based sewage treatment simulations such as the Semi Continuous Activated Sludge (SCAS) and Die Away (DA) systems to study aerobic biodegradation. NCT was used to determine the HPLC retention time required for effluent fractionation purposes. The mass spectrum and retention index of NCT TMS ether was used to guide a thorough search for the compound in the samples. The results showed that both the A-ring (loss of 4-^{14}C as $^{14}CO_2$) and side chain (loss of ^{14}C-26 as $^{14}CO_2$) of cholesterol underwent rapid oxidative cleavage. Apart from $^{14}CO_2$ as the major product, minor steroidal metabolites included cholest-3,5-diene and cholest-4-en-3-one were detected. Several other minor metabolites produced could not be conclusively identified due to the limits of detection of the radio chromatography methods and to dilution from unlabelled natural steroids which increased chromatographic loading. However, a cholestadiene (other than cholest-3,5-diene), a cholestadienol and a cholestenol were tentatively identified on the basis of the retention indices of authentic compounds and mass spectral data. There is some degree of doubt whether the two cholestadienes discovered were actual metabolites of cholesterol or were actually due to cholesteryl ester compounds undergoing thermal degradation in the GC injector. NCT was not identified as a major oxidative metabolite of aerobic bacterial degradation of cholesterol under the conditions used. For conversion to NCT by P_{450}-mediated systems cholesterol must undergo rearrangement of the Δ^5 double bond, cleavage of the C19 methyl and aromatisation of the A-ring, whereas herein cholesterol

underwent side chain cleavage, A-ring rupture, dehydration, dehydrogenation and isomerisation reactions.

The examination of anaerobic 4-^{14}C cholesterol metabolism was also studied. Over a 9-week incubation period the majority (*ca* 90 % maximum) of ^{14}C activity, was associated with SPM and showed a steady decline over the course of the incubation period. rHPLC fractionation of SPM extracts allowed detection of at least one metabolite. Total added 4-^{14}C-cholesterol burden in the extract was insignificant when compared to the total steroidal content which was considerable. In an attempt to remove a proportion of this background unlabelled steroids from the extract, rTLC was used prior to rGC analysis. Two radioactive components were identified in all extracts analysed by rTLC, of which one corresponded to the R_f of NCT. However, when analysed by rGC these radioactive fractions did not indicate the presence of 4-^{14}C NCT. Thus, NCT was not identified as a major metabolite of anaerobic cholesterol biodegradation herein.

The monitoring of STW effluents from Deephams and Harpenden discharging into the river Lea, North London, was conducted from February to August 1998. The analysis of liquid extracts showed that of the common estrogens, 17β-estradiol and estrone (*ca* 1ng L^{-1}) were present in both effluents, albeit at lower concentrations previously reported. The third natural estrogen, estriol (0.12 ± 0.08 ng L^{-1}) was also regularly identified in effluent samples from Harpenden STW. The synthetic estrogen, 17α-ethynylestradiol was below the limit of detection at both sites. The total average estrogen budget of 1.5 ng L^{-1} was lower than previously reported (Desbrow *et al.*, 1998). However, even at such low concentrations a dramatic effect in certain aquatic

fauna has been observed previously (Routledge *et al.*, 1998a). The lower concentrations reported herein were probably due to lower extraction efficiencies of aqueous samples, the time of day samples were taken and weather conditions and small differences in analytical methods. The phytoestrogen daidzein was found intermittently in liquid extracts from both STWs effluents but with an estrogenic activity *ca* 10^6 fold less active than 17β-estradiol the effect to the environment would probably be minimal. The natural sterols such as cholesterol, coprostanol and β-sitosterol were found consistently at both sites > 10 ng L^{-1}.

The natural estrogens, 17β-estradiol, estrone and 16α,17β-estriol, were not identified in solid extracts associated with the effluent, however, NCT was found in two samples, at a concentration of 39 ng L^{-1} and 136 ng l^{-1}, taken from Deephams on the same day. To examine the effluent for NCT the computed log K_{ow} value was used to estimate where in the effluent NCT was likely to be found. The value of 9.23 suggests that NCT is extremely hydrophobic and if present would probably be associated with the organic rich solid particulate matter of the wastewater effluent. Therefore, as well as aqueous samples, solid samples were also routinely taken for analysis. NCT was not found in SPM from Harpenden STW. The origins of the NCT in the SPM is as yet unknown. The radiolabelled cholesterol metabolism studies suggest NCT is unlikely to be derived from cholesterol metabolism during sewage treatment. There are a number of possible routes by which NCT could be biosynthesised. In mammalian systems A-ring aromatic estrogens are formed *via* the cleavage of the C19 methyl of 19-hydroxysteroids. Hence if 19-hydroxysteroid compounds exist in STW liquor (*ie* from influent or produced *in situ*) then the biosynthesis of NCT is plausible. To date there is no data on the existence of 19-hydroxysteroids in environments such

as sewage liquor. Cholest-1,4-dien-3-one, a metabolite of cholesterol and intermittently found in STW effluents herein, was used as starting material in the synthesis of NCT. Thus incubation studies of cholest-1,4-dien-3-one, rather than cholesterol, in sewage might prove interesting. It is clear from the radiolabelled cholesterol metabolism studies (herein and in the literature) that the alkyl side chain is extremely venerable to cleavage. NCT was not consistently identified in STW effluent samples and as such suggests that NCT may only be a transient intermediate, producing estrone (*via* side chain cleavage). This would help to establish why NCT was not found in all effluent samples analysed.

The organic rich SPM associated with STW effluent contained a suite of steroids, generally as the major components. The major sterol identified was coprostanol, β-sitosterol was generally present at greater concentrations than cholesterol.

6.2 Further work

Radiolabelled cholesterol (4 or 26-^{14}C) were used to determine aerobic metabolism during aerobic sewage treatment. Although four metabolites were identified as a result of cholesterol metabolism *ca* ten further products were not identified. Thus, the re-incubation and monitoring of radiolabelled cholesterol (at higher initial activity) metabolism would help in the identification of the unknown metabolites. Furthermore, the HPLC RT of cholest-3,5-diene should be determined. This would help to establish whether the cholestadienes reported herein were in fact metabolites of cholesterol or were due to thermal degradation during GC-MS analysis of more polar cholesterol metabolites.

The androstenone, 9α-hydroxyandrost-4-en-3,17-dione, has been shown to undergo B-ring rupture forming prior to A-ring aromatisation to produce 3-hydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione. Further metabolism results in A-ring rupture between C3 and C4. Herein, cholesterol was shown to undergo A-ring rupture with the cleavage of the 4-^{14}C as $^{14}CO_2$. The route by which cholesterol A-ring degradation occurs is as yet unknown but it is speculated that the degradation would be *via* the formation of cholest-4-en-3-one (identified herein) followed by B-ring rupture and A-ring aromatisation. Such compounds, similar in structure to *p-n*-nonylphenol, could have an estrogenic potential.

Further incubations with proposed intermediates such as coprostanol, 17β-estradiol and 16α,17β-estriol would enable a thorough examination of the processes occurring during sewage treatment and the effect on key compounds.

The examination of cholesterol metabolism during sewage treatment was greatly hindered in the present study due to high natural steroid burden of the activated sludge used. To eliminate competing compounds it would be advantageous to culture sewage microorganisms (anaerobic and aerobic) and use a single substrate (cholesterol, cholest-4-en-3-one and cholest-1,4-dien-3-one) as the sole carbon source. The incubation time should be comparable to true residence times of STW (*ie* 8 – 12 h).

Monitoring of STW effluents for continued examination of NCT, however, in conjunction influent samples should be taken and analysed to determine whether NCT (as the free alcohol or as a conjugate) is actually present in receiving waters or a product of metabolism during sewage treatment. Daidzein, found in STW effluents for

the first time, should be analysed in influent samples in order to determine removal rates. The preservative compounds classed as parabens, identified as having an estrogenic signature, have not previously been identified in either influent or effluent samples and thus could help to bridge the gap between chemicals which exhibit estrogenicity, their occurrence in nature and thus their importance to life.

The methodology for effluent monitoring should be reassessed. The initial effluent extraction procedure using ODS phase SPE cartridges was *ca* 50 % herein. The use of new phase material such as Waters Oasis®, using a hydrophilic-lipophilic balanced sorbent might increase extraction efficiencies, thus reducing the initial volume of effluent required and time. To increase HPLC fractionation efficiency a larger preparative column should be used enabling greater amounts of sample to be separated at anyone time. The use of fluorescence detection (directly or *via* derivatising with fluorescent label) may also increase sensitivity and reduce competition from unwanted compounds. Subsequent HPLC fractions should be examined by GC-MS using negative chemical ionisation (CI) mode to determine sensitivity compared with positive EI. The use of MS/MS mode should be assessed.

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Appendix A

Liquid Samples from Deephams
(ng L^{-1})

Date	Estrone	17β estradiol	Daidzein	Coprostanol	Cholesterol	NCT	Stigmasterol	β-sitosterol
09-Feb-98	3.4	0.66		176	53		22	73
24-Apr-98	0.3	0.24		75.7	15.5		23.2	30
15-May-98	0.2	0.40		48.9	10		12.1	19.5
07-Jul-98	3.1	0.98	2.73	155	29		32	12.3
20-Aug-98	0.5	0.27		207	39		48	82

Date and Time	Estrone	17β-estradiol	Daidzein	Coprostanol	Cholesterol	NCT	Stigmasterol	β-sitosterol
8/20/98 9:30	0.4	0.4		334	68		51	123
8/20/98 10:15	0.2	0.2		383	78		65	150
8/20/98 11:00	0.5	0.3		331	80		66	130
8/20/98 11:45	0.5	0.3		249	50		54	108
8/20/98 12:30	0.2	0.1		309	71		63	130
8/20/98 13:15	0.1	0.1		236	47		57	100
8/20/98 14:00	0.5	0.5		207	39		48	82
8/20/98 14:45	0.3	0.1		210	52		56	113
8/20/98 15:30	0.2	0.0		218	50		54	112
8/20/98 16:15	1.2	0.2		229	54		55	106

Solid Samples from Deephams using SPE
(ng L^{-1})

Date and Time	Coprostanol	Cholesterol	Stigmasterol	β-sitosterol	NCT
09/02/98 14:30	1010	540	144	909	39
09/02/98 14:30	1380	745	190	1065	136
22/04/98 14:00	1633	294	147	525	
13/05/98 13:40	889	170	73	332	
07/07/98 13:55	950	476	120	517	
20/08/98 14:45	826	430	221	973	

Date and Time	Coprostanol	Cholesterol	Stigmasterol	β-sitosterol	NCT
20/08/98 09:30	797	330	155	687	
20/08/98 10:15	435	184	154	466	
20/08/98 11:00	718	327	208	914	
20/08/98 11:45	547	284	157	660	
20/08/98 12:30	576	319	183	811	
20/08/98 13:15	698	315	198	741	
20/08/98 14:00	450	198	131	428	
20/08/98 14:45	826	430	221	973	
20/08/98 15:30	1230	546	254	1093	
20/08/98 16:15	1094	504	228	991	

Directly Taken Solid Samples from Deephams
($ng\ L^{-1}$)

Date	Coprostanol	Cholesterol	Stigmasterol	β-sitosterol	NCT
13/05/98 14:00	238	58	12	82	
07/07/98 11:22	107	46	16	87	
07/07/98 12:00	345	109	37	196	
07/07/98 12:45	164	53	15	91	
07/07/98 13:50	245	82	32	158	
07/07/98 14:10	100	26	8	54	

Directly Taken Solid Samples from Deephams
($ng\ mg^{-1}$)

Date and Time	Coprostanol	Cholesterol	Stigmasterol	β-sitosterol	NCT
13/05/98 14:00	616	150	28	212	
07/07/98 11:22	159	70	24.5	133	
07/07/98 12:00	410	130	44.5	234	
07/07/98 12:45	420	136	39	234	
07/07/98 13:50	360	121	48	232	
07/07/98 14:10	364	93	30	198	

Liquid Samples from Harpenden
($ng\ L^{-1}$)

Date	Estrone	17β-estradiol	Estriol	Daidzein	Coprostanol	Cholesterol	NCT	Stigmastero l	β-sitosterol	cholest-4-en-3-one	cholest-1,4-dien-3-one
20/05/98 14:00	0.6	0.1	0.1	1.65	625	81.5		19.7	84	6.7	6.5
2/8/98 14:10	0.5	0.1	0.2		2085	372		124	453		
2/8/98 15:10	0.3	0.1	0.05		2261	406		125	536		
2/8/98 16:00		0.2	0.1		1749	293		72	406		
2/8/98 7:05					1398	280		46	297		

Solid Samples from Harpenden from SPE
($ng\ L^{-1}$)

Date and Time	Coprostanol	Cholesterol	Stigmasterol	β-sitosterol	NCT
20/05/98 14:00	2530	263.5	46	330	
11/08/98 07:00	1120	245	60	305	
11/08/98 14:10	292	54	28	87	
11/08/98 15:10	716	112	57	201	
11/08/98 16:10	668	127	57	245	

Directly Taken Solid Samples from Harpenden
(ng L^{-1})

Date and Time	Coprostanol	Cholesterol	Stigmasterol	β-sitosterol	NCT
11/08/98 07:20	86	30	13	46	
11/08/98 08:00	197	73	33	127	
11/08/98 08:30	42	17	7	25	
11/08/98 08:50	27	12	4	14	
11/08/98 09:30	15	8	2	8	
11/08/98 11:40	25	9	3	21	
11/08/98 12:15	28	17	4	27	
11/08/98 12:50	11	7	2	12	
11/08/98 13:20	14	10	2	19	
11/08/98 13:45	6	3	1	7	
11/08/98 14:20	14	7	5	16	
11/08/98 14:50	3	2	1	4	
11/08/98 15:20	2	2	1	4	
11/08/98 16:15	15	4	2	5	

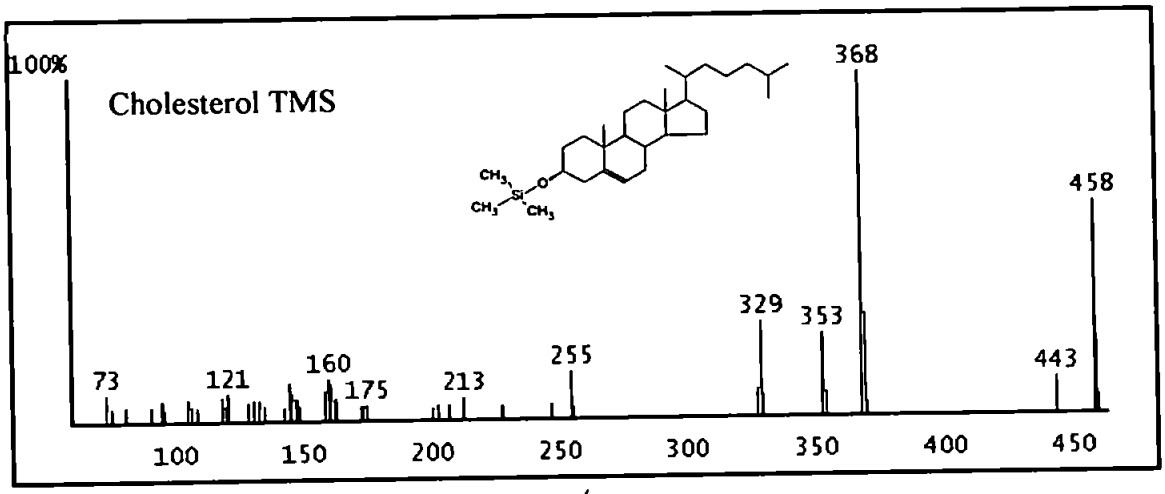
Directly Taken Solid Samples from Harpenden
(ng mg^{-1})

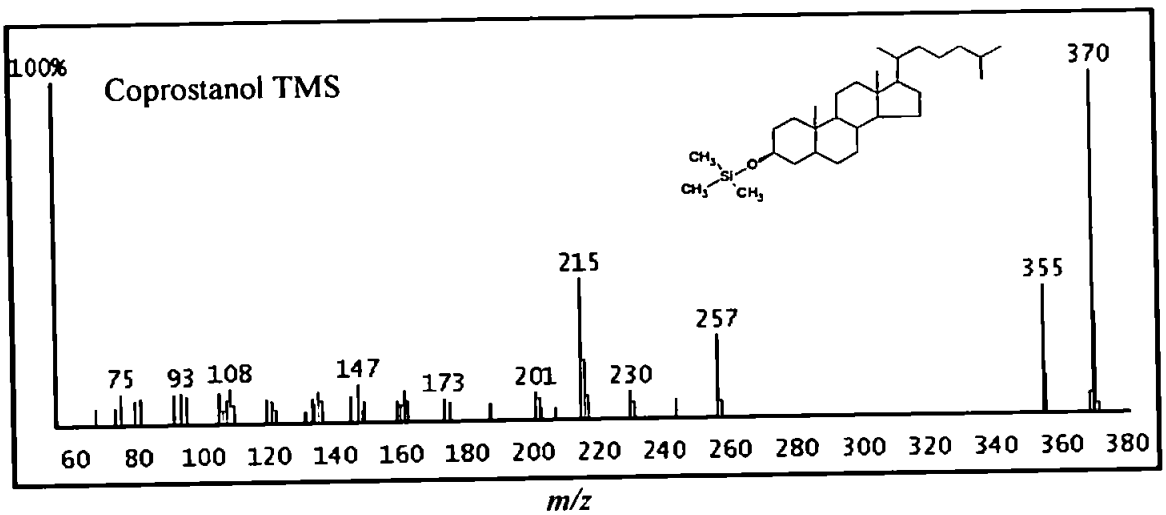
Date and Time	Coprostanol	Cholesterol	Stigmasterol	β-sitosterol	NCT
11/08/98 07:20	1100	389	166	592	
11/08/98 08:00	3939	1453	650	2540	
11/08/98 08:30	1908	748	315	1113	
11/08/98 08:50	1360	594	175	695	
11/08/98 09:30	436	248	72	250	
11/08/98 11:40	546	200	67	461	
11/08/98 12:15	793	470	118	777	
11/08/98 12:50	563	363	78	573	
11/08/98 13:20	232	160	30	309	
11/08/98 13:45	246	130	36	258	
11/08/98 14:20	185	97	61	219	
11/08/98 14:50	305	170	80	348	
11/08/98 15:20	240	175	50	435	
11/08/98 16:15	405	98	39	144	

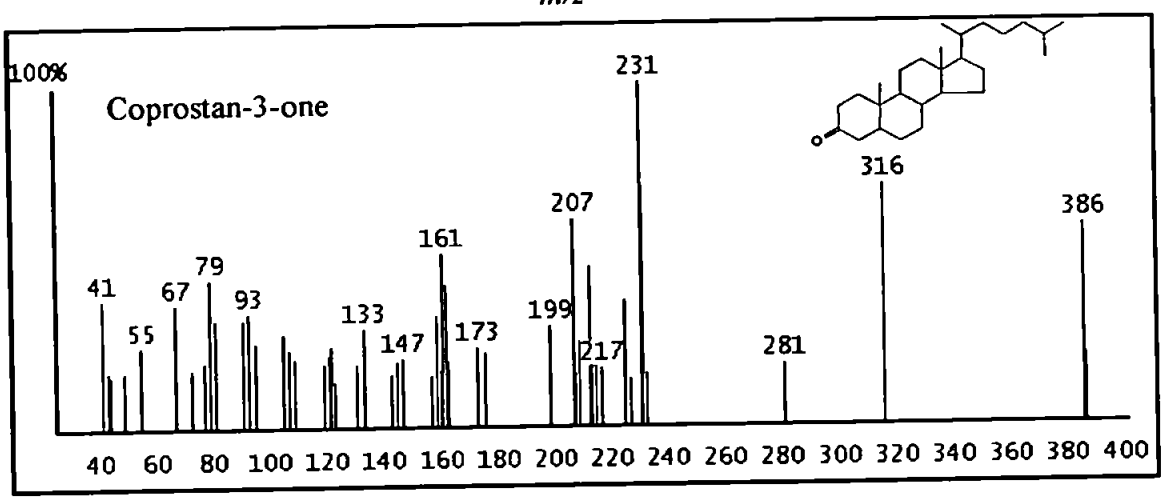
Note: all steroid concentrations (except 17β-estradiol) were determined by response factors relative to the external standard, 1,4-^{2}H-17β-estradiol, as the TMS ether derivative. Natural occurring 17β-estradiol concentrations were determined by linear regression.

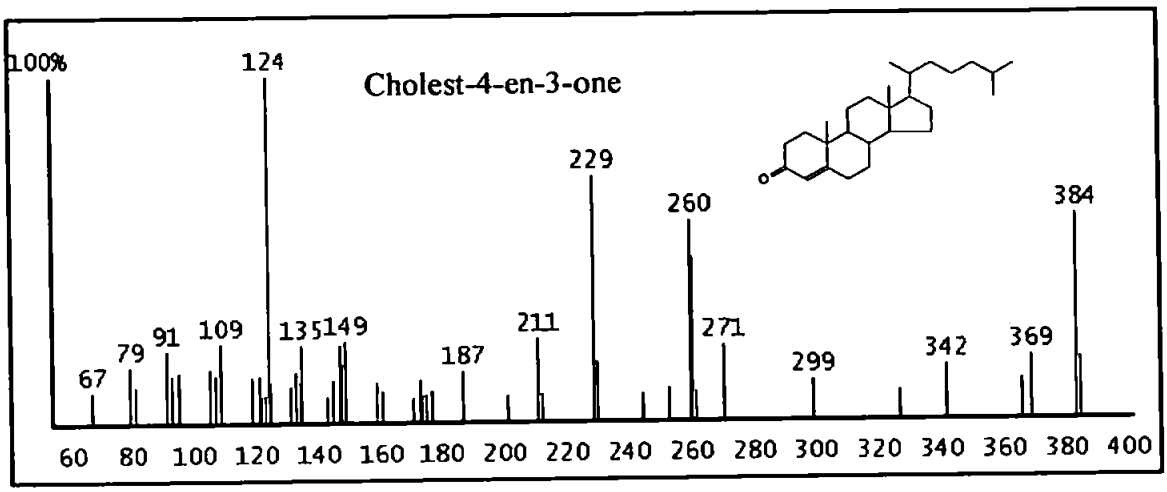
Appendices B

Mass spectra of steroids investigated





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Cholest-4-en-3-one
229
260
384
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149
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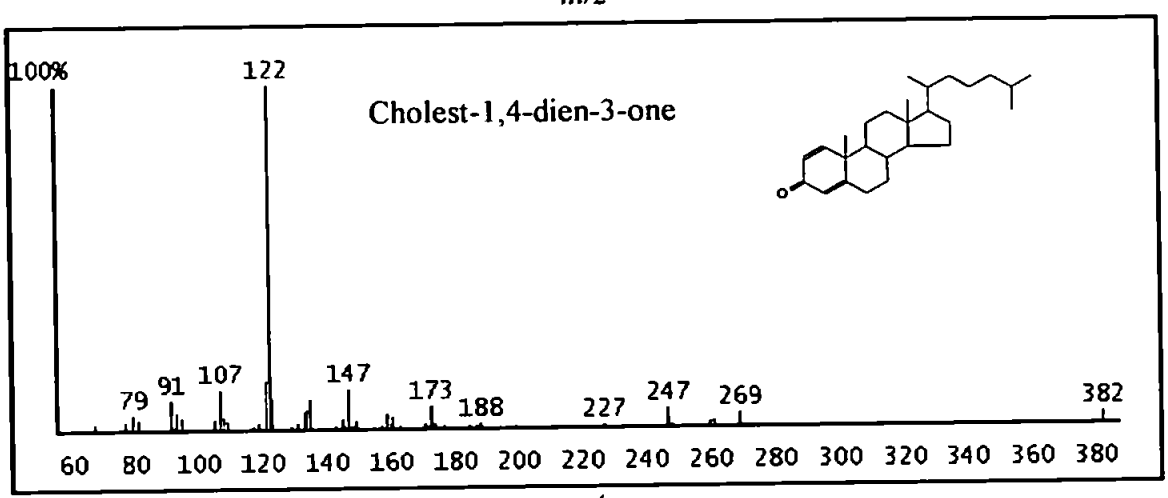
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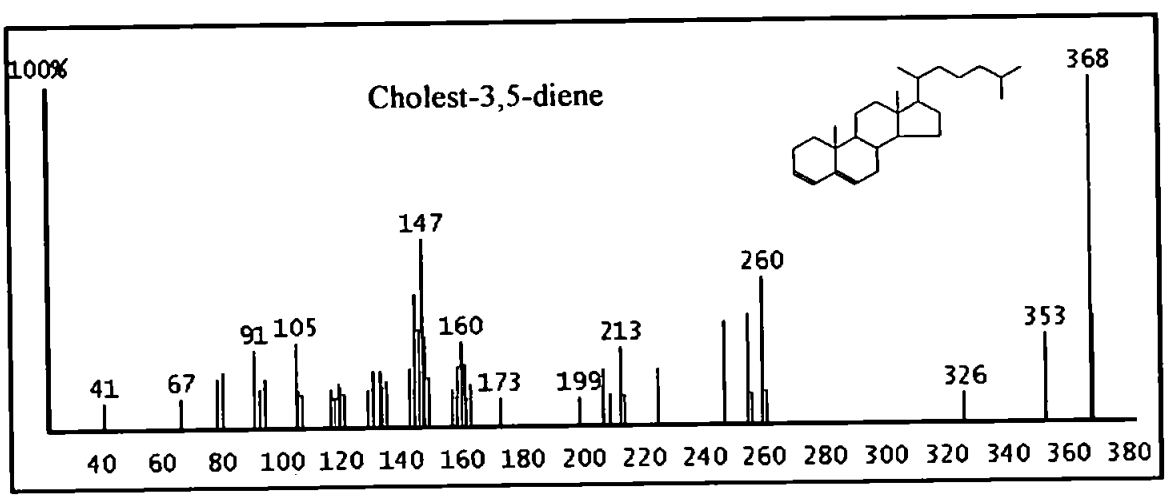
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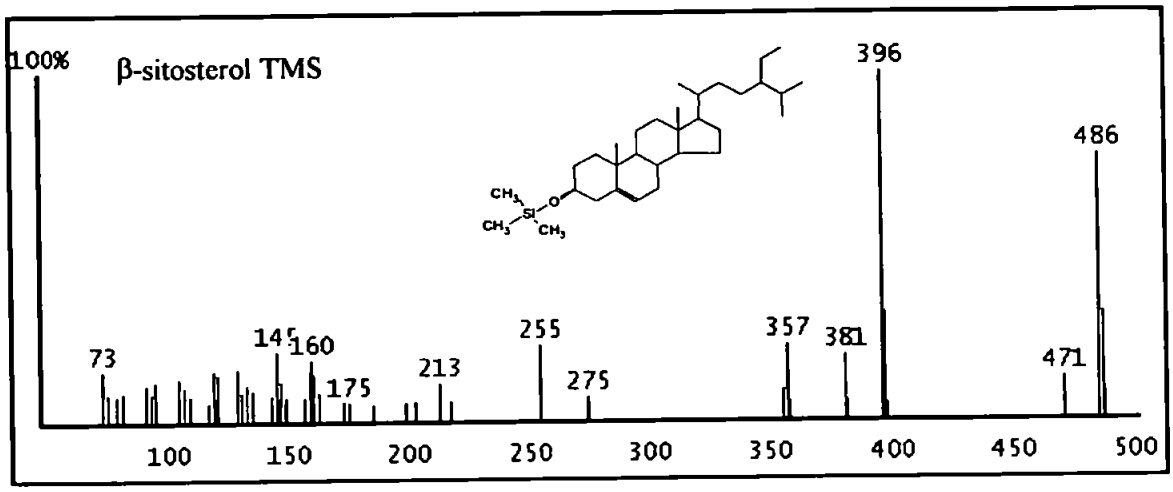
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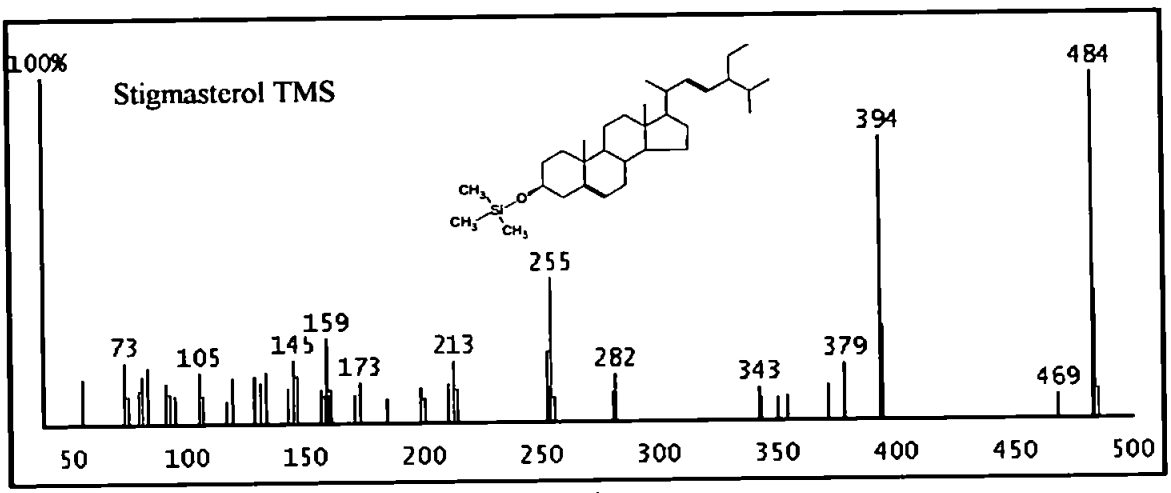
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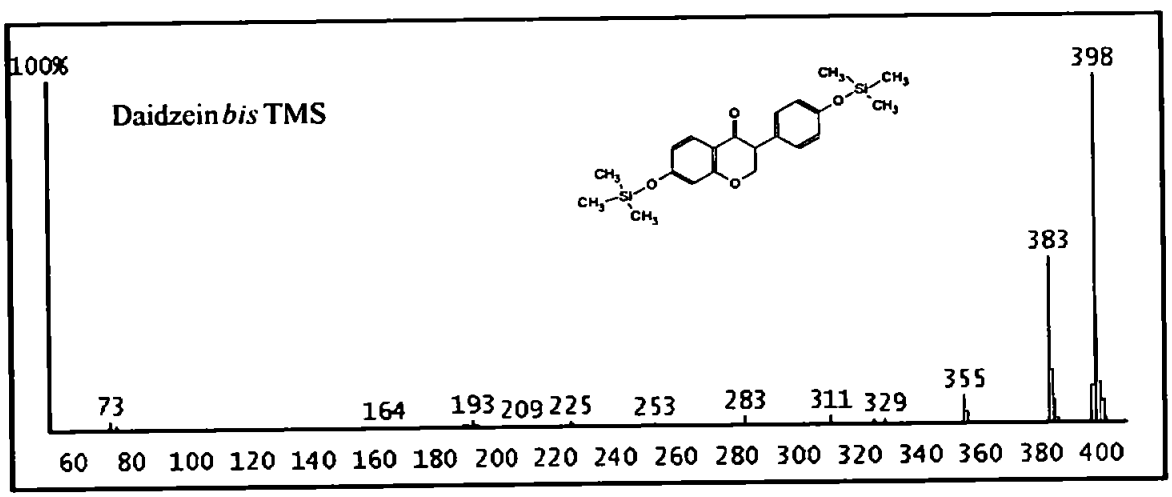
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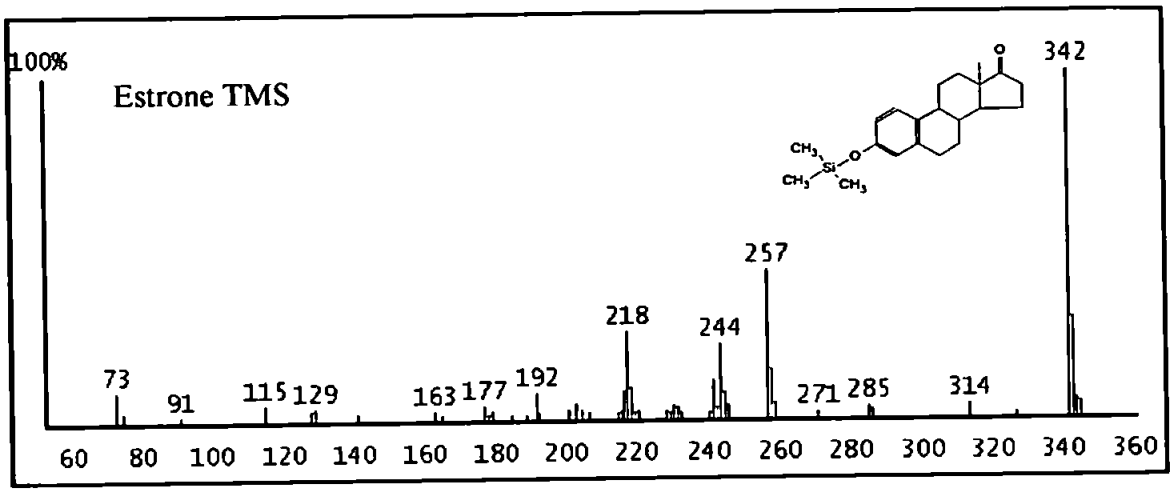
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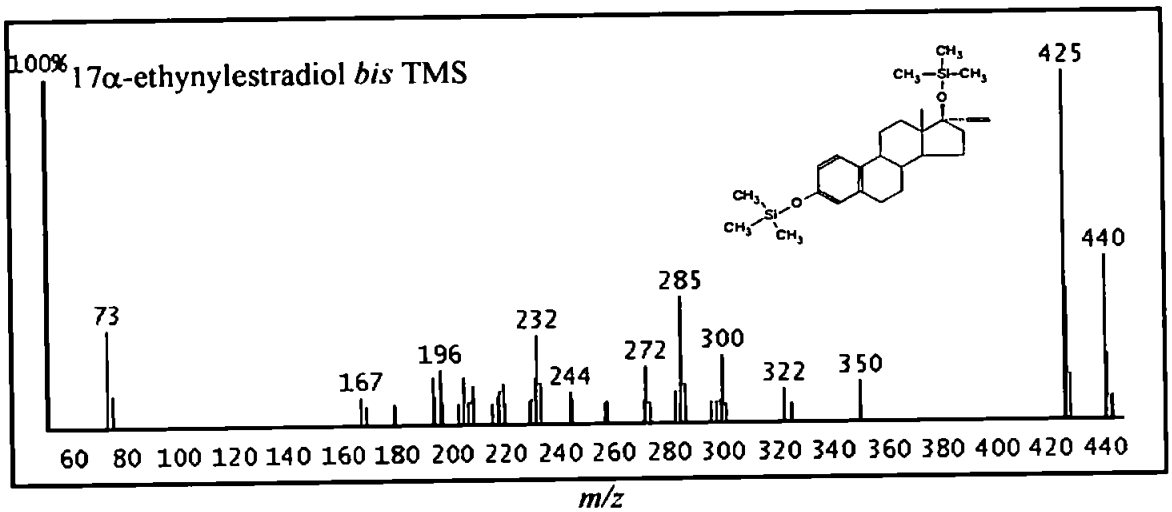
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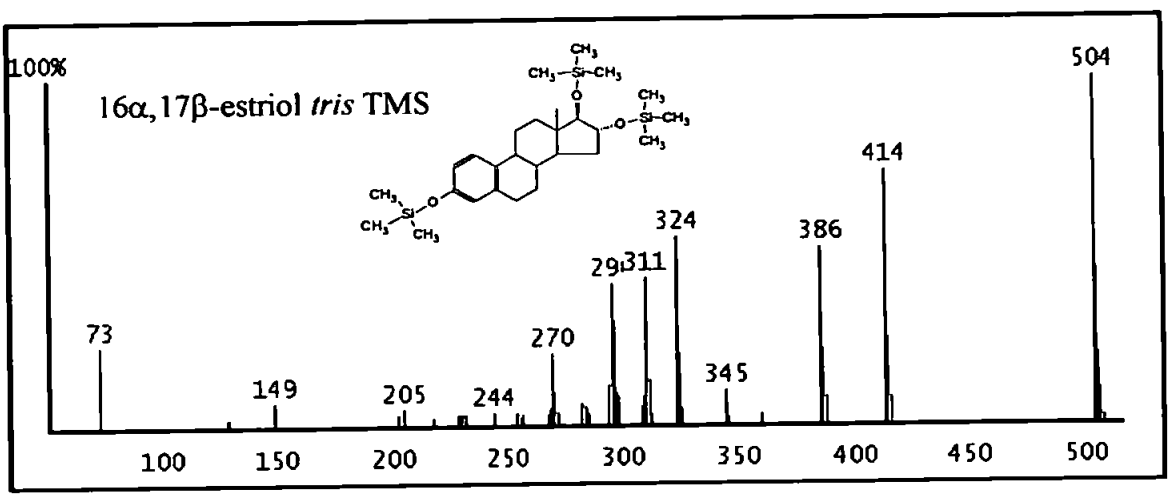
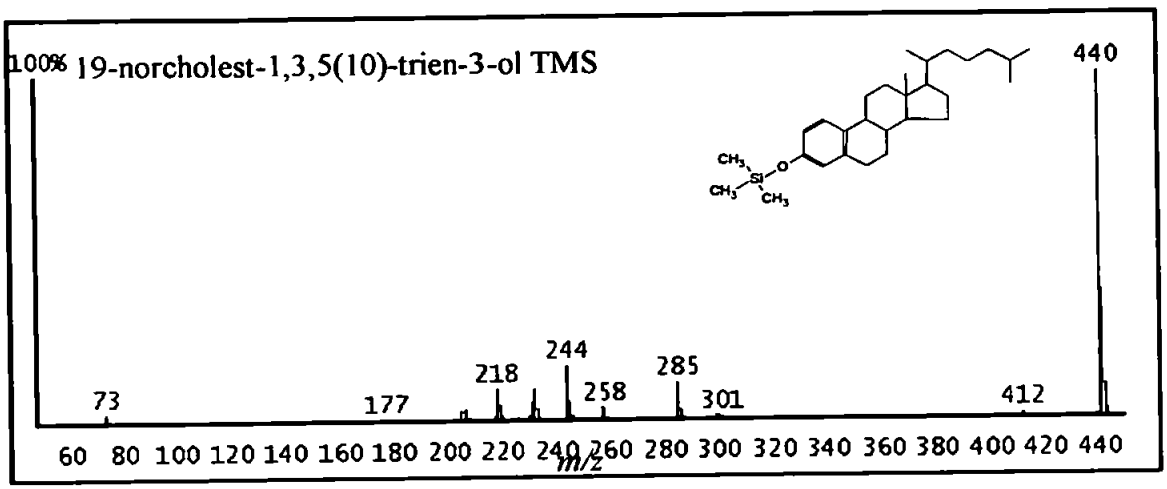
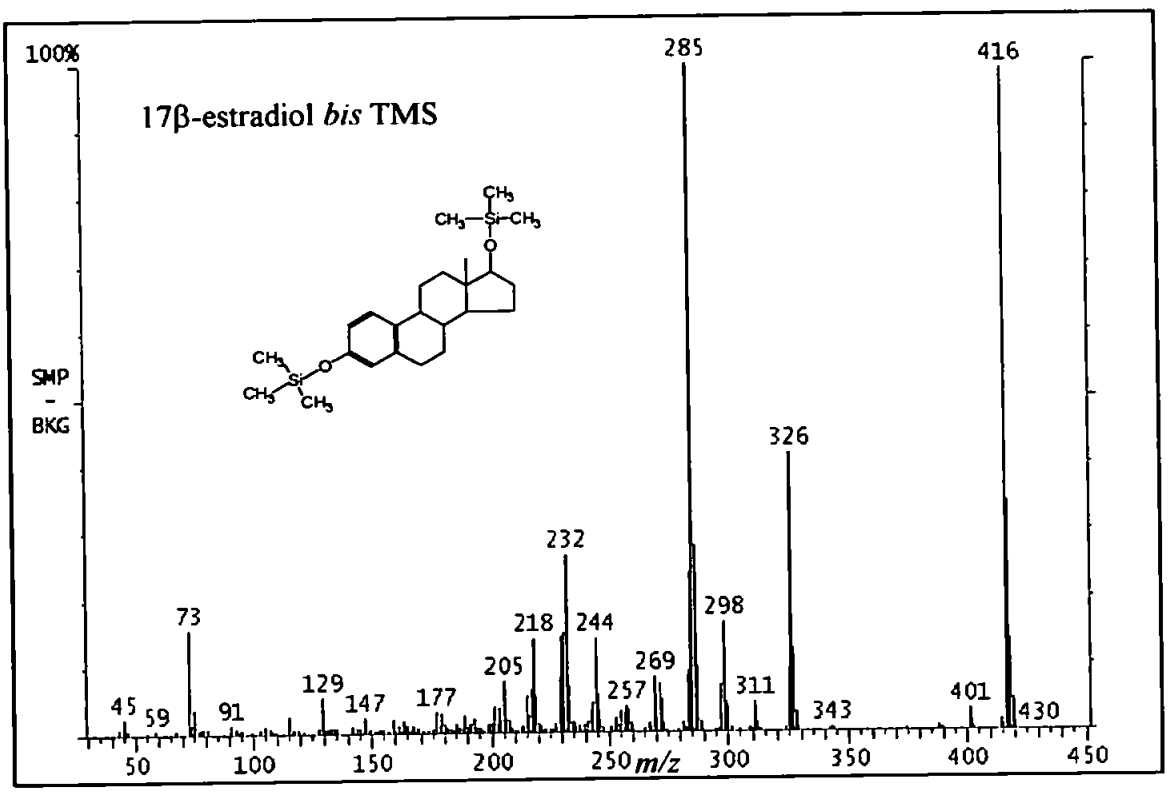
m/z


100%
16α,17β-estriol *tris* TMS
504
414
324
386
291
311
73
270
345
149
205
244
100 150 200 250 300 350 400 450 500
m/z


100% 19-norcholest-1,3,5(10)-trien-3-ol TMS
440
73
177
218
244
258
285
301
412
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440
m/z


100%
17β-estradiol bis TMS
SMP
BKG
285
416
326
232
73
218
244
298
205
269
129
257
311
147
177
401
45
59
91
343
430
50 100 150 200 250 m/z 300 350 400 450